

**Candidate Gene Expression and SNP Analyses of Toxin-Induced Dilated
Cardiomyopathy in the Turkey (*Meleagris gallopavo*)**

By

Kuan-chin Lin

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Dr. E. J. Smith, Chair

Dr. M. Potts

Dr. Z. Tu

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ABSTRACT

Dilated cardiomyopathy (DCM), a heart disease, affects many vertebrates including humans and poultry. The disease can be either idiopathic (IDCM) or toxin-induced. Idiopathic DCM often occurs without a consensus cause. Though genetic and other studies of IDCM are extensive, the specific etiology of toxin-induced is still unknown. Here, our objective was to compare the level of mRNA expression of two candidate genes including troponin T (cTnT) and phospholamban (PLN) using quantitative reverse transcription polymerase chain reaction (RT-PCR) in toxin-induced DCM affected and unaffected turkeys. Cardiac TnT and PLN were chosen because their spontaneous expression has been reported to be associated with IDCM. We also scanned these genes for single nucleotide polymorphisms (SNPs) that could be useful in evaluating their functions in the incidence and severity of toxin-induced DCM in turkeys. There were no significant differences between affected and unaffected birds in the expression of both cTnT and PLN. A total of 12 SNPs were detected in cTnT and PLN DNA sequences. One of the seven haplotypes detected in cTnT was the most frequent. Linkage analysis showed that cTnT gene was unlinked on the current turkey genetic map. Resources developed here, including SNPs, haplotypes, cDNA sequences, and the PCR-RFLP genotype procedure will be used for future investigations involving cTnT and PLN and toxin-induced DCM.

Keywords: Turkey, Toxin-induced Cardiomyopathy, Troponin T, Phospholamban, RT-PCR, PCR-RFLP, SNPs

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CHAPTER 1

INTRODUCTION

The turkey, *Meleagris gallopavo*, is one of the most important food animals in the United States of America (US) and other developed countries. In total production output and farm receipt, it ranks second only to chicken. Despite this importance as a major meat source, the turkey genome and genetic basis of many of its economically important traits remain poorly understood. An increased understanding of the turkey genome could facilitate the identification of genes for traits that are important to continued growth in the turkey industry.

In the past three decades, turkey production in the U.S. has increased by more than three-fold. Preliminary annual statistics for 2005 estimate that the total US production of turkeys about 257 million birds. Though the 2005 estimates are currently not publicly available, data from previous years indicate that the total annual receipts or income from the turkey industry in the US exceeds \$7.8 billion. This gross receipt includes both the increased consumer demand in the US and sales from turkey meat and meat-related products abroad. It is estimated that the total annual export of turkeys exceeds 400 million pounds. It is therefore not surprising that this rapid growth in the use of turkey and turkey products has contributed significantly to the unparallel rise of the poultry industry.

In order to meet consumer demand, turkeys have been selected for rapid growth and higher average body weight at market age. This has led to the speculation that, as in other animals, increased physiological abnormalities and other health problems, such as circulatory disturbances may be a result of this rapid growth of the turkey

(<http://www.eatturkey.com/consumer/stats/stats.html>;
<http://www.census.gov/Press-Release/www/2005/cb05ff-18-2>).

In commercial turkeys, a prevalent circulatory problem is dilated cardiomyopathy (DCM) (Frame et al., 1999). Dilated cardiomyopathy is a myocardial disease that is characterized by enlarged ventricles, cavity dilatation, and systolic and diastolic dysfunction (Fatkin and Graham, 2002). Affected young poulets have ruffled feathers, drooping wings, and unthrifty appearance (Sautter et al., 1968). Clinical symptoms of DCM including dyspnea, weakness, and edema have been reported to be associated with heart failure which if severe may cause sudden death, resulting in economic loss (Fatkin and Graham, 2002).

In commercial turkeys, it has been estimated that DCM causes early death at a rate of 2 to 4% as well as weight loss in birds between 2- and 4-weeks-of-age (Frame et al., 1999; Zepeda and Kooyman, 2002). In humans, DCM affects approximately 4.7 million people and \$17.8 billion health-care cost annually in the U.S. (Schmitt et al.,

2003). In addition to the turkey and human, DCM has also been reported in other important farm animals including chicken and cattle (Wu et al., 2003 and Nart et al., 2004a).

Though the etiology of DCM is poorly understood, in general, factors that have been implicated in the incidence and severity of DCM can be either genetic or environmental. The environmental factors include nutrition, management, pathogens, stress, and toxins (Frame et al., 1999; Poller et al., 2005). Furazolidone (Fz), a drug normally used to treat enteritis and diarrhea, has been shown to induce DCM in turkeys at toxic levels (Ali, 1989; White, 1989). Young birds, five weeks-of-age or younger, fed diets containing 700 parts per million (ppm) of Fz for two to three weeks, develop DCM (Genao et al., 1996). Characteristics of affected birds include increased heart volumes, left ventricular dilation, and fractional shortening as well as altered membrane transport (Hajjar et al., 1993). In gross morphology, these characteristics are similar to those seen in birds and humans affected by spontaneous DCM. Additional similarities include altered Ca^{2+} metabolism and the beta receptor-adenylyl cyclase signaling system. These similarities indicate that DCM could be a useful experimental model for understanding spontaneous DCM in humans and other animals (Hajjar et al., 1993; Genao et al., 1996).

As a model system, Fz-induced DCM has been extensively investigated

resulting in increased knowledge about many aspects of toxin-induced DCM. It is known, for example, that the toxic level that causes significant mortality but not death of all of the birds in a particular flock is 700 ppm (Gyenai, MS thesis, 2005). The genetic basis of susceptibility to the toxic effect of Furazolidone has been very little studied. However, the genetic mechanisms that influence IDCM have been very widely investigated (Dolf et al., 1998; Alroy et al., 2000; Biesiadecki et al., 2002; Fatkin and Graham, 2002).

The investigations of the genetic basis have included identifying candidate genes. From these investigations, at least 18 genes have been reported to influence spontaneous DCM including phospholamban (PLN) and cardiac troponin T (cTNT). The phospholamban gene is involved in regulating calcium uptake in the sarcoplasmic reticulum. Mutations in PLN, as expected, have been reported to affect calcium transport in cells leading to abnormal myocardial function (Liew and Dzau, 2004). Swinger et al. (1995) used western and northern blot analyses to compare protein and transcripts levels, respectively, of PLN in human non-failing and failing heart tissues. They reported that in DCM-affected heart, PLN transcripts, but not protein levels, were lower.

Like PLN, cTnT is a candidate gene for DCM because of its role in myofibrillar calcium sensitivity. Mutations in cTnT change the sensitivity of

myofilaments to calcium. In both turkey and human hearts, abnormal splicing of multiple exons of cTnT has been associated with the incidence of DCM (Biesiadecki et al., 2004). A single nucleotide polymorphism (SNP) is believed to be responsible for the alternative splicing observed in affected birds (Venkatraman et al., 2005). In cattle, Dolf et al. (1998) described a biallelic locus, possibly a SNP, responsible for DCM shown previously to have autosomal recessive inheritance.

More recently, Liew and Dzau (2004) reported that genome-expression profiling provides an opportunity to identify molecules with the potential of becoming possible clinical tests to predict the early stage of heart failure in animals. Additionally, gene expression is a useful approach for detecting specific genotypes associated with disease phenotypes. While SNPs influence gene expression, their utility in the identification of disease genes is primarily through linkage disequilibrium (Suh and Vijg, 2005).

Work in our laboratory, also the focus of my thesis research, has as its long-term goal the identification of genes that influence toxin-induced cardiomyopathy. This thesis research extends previous investigations designed to contribute to this overall goal and provides resources that could be used in other investigations in the lab for reaching this main objective.

A completed investigation that provides useful foundation for this thesis work

involved the development of parameters for efficient use of echocardiography as a non-invasive technique for identifying DCM-affected birds at 0 to 4 weeks of age. Another investigation involved a determination that turkey strains differ in their susceptibility to the toxic levels of Furazolidone (Gyenai, MS thesis, 2005). Based on the observed mortality rate, my research mainly focused on examining the differences in expression of cTnT and PLN genes, between normal and Fz-induced DCM in turkey poulets. Here, we hypothesized that there are no significant differences in the expression of PLN and cTnT genes between turkeys affected or unaffected by DCM induced by furazolidone feeding. This can be expressed as $\mu_{RNAdem} = \mu_{RNAnormal}$, where μ is the average mRNA level as determined by quantitative real time polymerase chain reaction (RT-PCR).

Since others have shown that spontaneous DCM is affected by PLN and cTnT expression, it is rationale that my search for candidate genes for toxin-induced DCM in the turkey includes these two genes. Here, I will evaluate whether the genetic of toxin-induced DCM basis is partly due to variation in expression of candidate genes PLN and cTnT. Specific aims for the thesis research include:

1. Use RT-PCR to compare the expressions of cTnT and PLN candidate genes for DCM in normal and DCM-affected turkeys after exposure to toxic level of Furazolidone.

2. Conduct SNP analysis to identify informative SNPs and haplotypes in a parallel investigation of the role of cTnT and PLN genes in susceptibility or resistance to toxin-induced DCM.
3. Use SNPs to map candidate genes on the turkey genetic map.

This project provides a foundation for our laboratory to use SNP analysis and genomic tools to further characterize the genes evaluated in the present work and to identify others that may affect the incidence of DCM in the turkey.

CHAPTER 2

LITERATURE REVIEW

Introduction

The incidence of dilated cardiomyopathy (DCM) or round heart disease in turkeys can be considered a random event. Sudden death from DCM causes significant economic losses in the poultry industry. In mammalian species, such as humans, DCM is reported to be a major cause of congestive heart failure, which also causes extensive economic losses every year. Recently, at least 18 candidate genes were reported to be involved in DCM, including cardiac troponin T and phospholamban, which are being considered for developing potential genetic markers for DCM. However, the understanding of genetically based DCM in the turkey remains little. This thesis will examine published works related to DCM in turkeys and other varieties.

2.1 Turkeys

Turkeys, *Meleagris gallopavo*, are one of the most widely distributed game birds that are indigenous to North America. They prefer hardwood and mixed conifer-hardwood forests for habitats. In the classification, turkeys belong to the order *Galliformes* and the family *Meleagrididae*. In addition to *gallopavo*, there are six other subspecies, *silvestris*, *osceola*, *merriami*, *intermedia*, *oneusta*, and *mexicana*. These subspecies are distinguished by geographic range and morphological differences in size

and plumage. Three of the seven subspecies, *gallopavo*, *silvestris*, and *merriami*, are important for domestication. The domesticated turkey primarily descends from the species *gallopavo*, and later, the subspecies *silvestris* was hybridized with the *gallopavo* to form commercial turkeys (Crawford, 1992; Eaton, 1992).

The recognized varieties of *Meleagris gallopavo* include Narragansett, Bronze, Black, White Holland, Beltsville Small White, Slate, Royal Palm, and Bourbon Red. Modern commercial turkeys were developed from Bronze and White Holland varieties through mating and selection (Austic and Nesheim, 1990). Other than differences among the eight varieties for plumage color our understanding of their genetic variation and differences remains little. Recently, Smith et al. (2005) used diverse DNA markers including randomly amplified polymorphic DNA (RAPD), microsatellite, and expression sequence tags-based (single nucleotide polymorphisms) to investigate the genetic relatedness among five of these turkey varieties. Their results suggested that on average Narragansett and Royal Palm are much more closely related.

Both commercial and non-commercial turkeys suffer from diseases and abnormalities that lead to economic loss in the poultry industry (Hunsaker, 1971; Sautter et al., 1968). Generally, different stresses including nutrition, management, pathogens, and parasites are responsible for avian diseases that affect the respiratory system such as newcastle disease and cardiomyopathy. Besides the common respiratory

problems, cardiomyopathy has been also of interest to scientists and researchers.

Cardiomyopathies reported in mammals are classified into four categories: hypertrophic, dilated, arrhythmogenic right ventricular dysplasia, and restrictive. Dilated cardiomyopathy, the focuses of my thesis research, often leads to sudden death and depressed growth rates in the turkey (Mirsalimi et al., 1990; Fatkin and Graham 2002; Mogensen et al., 2004). Hunsaker (1971) examined four hundred turkey poult's and found 50% incidence of dilated cardiomyopathy in four strains. The total mortality varying from a maximum of 10% to minimum of 5% was observed among the four strains. Additionally, more males than females were affected in each strain. But, the genetic basis understanding of DCM in turkeys is poor.

2.2 Dilated cardiomyopathy

Dilated cardiomyopathy, a lethal disease, is a cardiac disorder that results in enlargement of the heart chambers. Histologically, a DCM affected heart shows myofibrillar lysis, glycogen accumulation, sarcoplasmic reticulum proliferation, and loss of mitochondria (Mirsalimi et al., 1990; Mogensen et al., 2004). In general, DCM can be spontaneous or inducible. Spontaneous DCM is often considered to be idiopathic dilated cardiomyopathy (IDCM). Cardiac dysfunction due to IDCM can cause weakened hearts and ineffective pumping and arrhythmia, thromboembolic events, and congestive heart failure (Tidholm and Jonsson, 2005; Mohan et al., 2002; Czarnecki,

1984). Severe congestive heart failure could result in sudden death and cause economic loss. Approximately 20 % of the mortality rate of human congestive heart failure is due to DCM (American Heart Association, 2006). In turkey poulets, about 5 % of mortality rate is due to spontaneous DCM (Zepeda and Kooyman, 2002). Dilated cardiomyopathy has also been reported in cattle of Holstein-Friesian breed in the past two decades (Nart et al., 2004b).

The specific cause of death due to IDCM is unknown. An IDCM may result from a combination of factors such as genetics, nutritional deficiencies, metabolic disorders, enzyme disorders, immunologic abnormalities, infectious diseases, stress, and drug-, toxin-, and tachycardia-induced hypokinesis (Mohan et al., 2002). The most common cause IDCM, however, is genetics. Genetic studies of hereditary DCM indicate that DCM is a heterogeneous disease. Murphy and Starling (2005) indicated that up to 50% of IDCM in humans is inherited. Several modes of inheritance of DCM have been reported including autosomal recessive, autosomal dominant, X-linked, and mitochondrial. Infantile DCM may be due to autosomal recessive inheritance (Alroy et al., 2000). In cattle, Dolf et al. (1998) used segregation analyses under three models to implicate that a single biallelic major locus in the autosomal recessive inheritance of DCM. In humans, autosomal dominant mutations in genes such as lamin A/C (*LMINA*) cause a conduction-system disorder were reported to be autosomal dominant. X-linked

genes include Dystryophin (*DMD*), which encodes a large cytoskeletal protein, and Tzfazzin (*G4.5*) gene, which encodes a group of proteins of unknown function with no similarities to other proteins. Mutations in *DMD* were first identified in Duchenne and Becker muscular dystrophies. Additionally, *DMD* mutations also been found to cause X-linked DCM affected in males to lead to death within 1-2 year. Mutations in *G4.5* genes were first associated with Barth's syndrome, which also found in inherited DCM families and caused X-linked recessive disorders. Point mutations and deletions in mitochondrial DNA have been found in multi-system disorders associated with cardiac abnormalities including DCM (Fatkin and Graham, 2002).

Inherited DCM has also been attributed to multi-genes including mitochondrial genes (Olson and Keating, 1996; Alroy et al., 2000). Recently, at least 18 genes were identified to be candidate genes involved in DCM including cardiac troponin T (*cTnT*) and phospholamban (*PLN*). Cardiac troponin T gene encodes cTnT protein, a sarcomere thin filament that plays an important role of adjusting sensitivity of the troponin complex to calcium during cardiac relaxation and contraction. Though deletions in *cTnT* have been associated with IDCM in humans, the association of mutations in troponin I and alpha-tropomyosin genes with human IDCM was not consistent in cases (Fatkin and Graham, 2002; Mogensen, et al., 2004). In addition to human, abnormal splicing of multiple exons of *cTnT* has been described in animals

with DCM including turkey, dog, cat, and guinea pig. Osterziel and Perrot (2005) indicated that *cTnT* mutations in IDCM patients were found as early as one month of age.

The *PLN* gene, implicated in DCM in various species, encodes PLN protein that regulates the sarcoplasmic reticulum (SR) Ca^{2+} pump and controls the size of SR Ca^{2+} store during diastole. Fortunately, DNA sequences of *PLN* of different species including human, chicken, rabbit, rat, and mouse, are also available in public databases. These have been used for both comparative and expression studies McTiernan et al. (1999), for example, described a marked conserved region of about 217 bp upstream of the transcription start site. Schwinger et al. (1995) and MacLennan and Kranias (2003) used Northern blot analysis to show significant differential expression between non-failing and failing human hearts caused by IDCM. The transcript level decreased by at least 30% in failing hearts. Schwinger et al. (1995), however, did not show any significant differences in the protein levels. Despite its importance in DCM and other conditions, our knowledge of the *PLN* gene in the turkey is negligible. In this thesis, the *PLN* gene will be potentially developed and characterized.

World-wide, approximately 23 million people suffer from congestive heart failure, and 2 million new cases of the disease are reported each year. But, the understanding of this disease's molecular mechanism is still incomplete (Tandon, 2005).

Because DCM is believed to be the most common cause of congestive heart failure, some animal models have been developed to help us further study and understand the molecular mechanisms. Such animal model has its advantages and disadvantages. The use of any of them as a model is influenced by ethics and economical considerations, accessibility, and reproducibility (Hasenfuss, 1998; Wu et al., 2003; Tidholm and Jonsson, 2005). Most animal models are not suitable because some characteristics, such as diseased myocardium, the sensitivity of myofilaments, and pathophysiology between non-failing and failing hearts appear to be different from human condition (Hajjar, et al., 1993).

Rodent models such as rat, mice, and rabbit are relatively inexpensive with the advantage of a large sample size and short gestation period. Some can also be easily genetically manipulated, such as several transgenic models of heart failure established from deletion or addition of genes. For instance, gene-targeted disruption of the muscle LIM protein (MLP), a regulator of myogenic differentiation, in mice is a model of DCM and heart failure. Mice knocked out the *MLP* gene will develop DCM associated with myocardial hypertrophy (Hasenfuss, 1998). These small animal models, however, have the disadvantage that they can not be physiologically monitored for some syndromes associated with DCM. For example, in transgenic mice, clinical signs of congestive heart failure were not obvious after beta-adrenergic receptor kinases gene

was disrupted (Hasenfuss, 1998; Traystman, 2003). Additionally, the physiological events of DCM and congestive heart failure in these small animal models are less similar than these in large models, when both are compared with human IDCM. For instance, resting heart rates of small animal models are much higher and left ventricular functions and volumes are not as accurate as large animal models for the study (Kapoor, 2005).

There are other limitations indicated that some of small animal models fail as suitable models for studying human DCM. In the rat model, for example, a very short action potential lacking a plateau phase exhibits in myocardium. The activity of the sarcoplasmic reticulum calcium pump can predominate calcium removal from the cytosol whereas $\text{Na}^+ / \text{Ca}^{2+}$ -exchanger activity is less relevant. These differences implicate that the rat model is less appropriate as a model for studying heart failure and DCM (Hasenfuss, 1998; Kapoor, 2005). On the other hand, DCM in large animal models, such as dog, cat, pig, and cattle, has been shown to be similar to the human conditions in clinical, pathological, genetic, biochemical, or morphologic characteristics for studying DCM and heart failure under induced or spontaneous conditions (Hendrick et al., 1990; Hasenfuss, 1998; Alroy et al., 2000; Kapoor, 2005). However, large animals are costly, have longer life span and require substantial resources with respect to care and housing (Hasenfuss, 1998; Kapoor, 2005).

Another option for studying human DCM and heart failure is the turkey (*Meleagris gallopavo*) as a model. The turkey has been used to test the effect of several toxins including furazolidone (Fz), propranolol, and digoxin. Furazolidone is reported to significantly affect the dilation of hearts (Gwathmey and Hamlin 1983) and to induce DCM (Magwood and Bray, 1962). The mechanism by which Fz induces DCM in the turkey is not fully understood. It is speculated that Fz acts against converting pyruvate to acetyl coenzyme A may be inhibited by some compounds (Czarnecki et al., 1975). However, this drug initiates the effects in the membrane system if the myocardial cell and causes a result of enzyme inhibition. The inhibition then leads to alteration of the mitochondrial and myofibrillar components with concomitant increase in cytoplasmic glycogen. Furazolidone-induced DCM in turkey causes clinical symptoms, like cardiac hypertrophy and congestive heart failure. Other characteristics of Fz induced DCM include myelin fibers and glycogen deposits in the mitochondria of the right ventricular wall of affected turkeys (Czarnecki, 1979). Mirsalimi (1990) reported biochemical changes increased calcium-transport ATPase activity of the sarcoplasmic reticulum in Fz-induced. Their work also showed depressed mitochondrial activity due to the reduction of 3-hydroxyacyl CoA dehydrogenase (HADH, the fatty acid oxidation marker) and cretin kinase (CK, Krebs cycle marker). Further, reduced glycolytic activity as indicated by lower activities of phosphofructokinase (PFK) and lactate dehydrogenase

(LDH) was also observed. These enzymes are thus potential biochemical makers for detecting DCM.

Hajjar et al. (1993) discussed the similarities and differences in pathophysiological and biochemical characteristics between Fz-induced DCM and human IDCM. The six major similarities include decreased ejection fraction, increased end diastolic volume, gross morphology, histopathology, muscle physiology, noradrenaline, and myofilaments. These similarities indicate that the Fz-indcued DCM in the turkey is a suitable model for studying human DCM.

Using Fz-induced DCM in the turkey as a model for human DCM, Gwathmey et al. (1999) tested the efficiency of Carteolol, a beta-adrenergic blocker. A total of 59% mortality was observed in the nontreated DCM group, 55% mortality in the group treated with low dose of Carteolol, and 22% mortality in the group treated with high dose of Carteolol. They also reported that activities of SR Ca^{2+} -ATPase, myofibrillar ATPase, cretin kinase, lactate dehydrogenase, and aspartate transaminase, all of which have been implicated in DCM were restored. Additionally, the beta-adrenergic receptor was increased in birds on Carteolol. Other observations included ejection fraction and left ventricular peak systolic pressure. Washington et al. (2001) also reported similar results of improved myocardial contractility, including heart rate in Fz-induced turkeys after treatment with carteolol. These results are

consistent with those in human. Matsumori et al. (1992) showed improvement in 57% of DCM patients on Carteolol. The similarities in improvement in human DCM and Fz-induced turkeys indicate the value of the Fz-induced turkey as a successful model for evaluation potential therapies for DCM in human.

Due to the severe health burden from DCM, scientists and policy members have a strong interest in finding better diagnostic techniques and treatments (Mohan et al., 2002). Molecular diagnosis provides a strong opportunity, including the use of gene expression profile and SNPs for better and earlier diagnosis.

2.3 Gene expression

Differences in gene expression are responsible for differences in cellular function and phenotype, and many represent responses to environmental stimuli and perturbations (Cho et al., 1998). In general, transcripts are the preferred measures of gene expression. Protein-based approaches of measuring gene expression are more difficult and less sensitive than methods that involve determining mRNA levels. The latter are more informative about the activity of genes and cell state. As far as most genes are concerned, changes in RNA abundance are related to changes in protein abundance (Lockhart and Winzeler, 2000). By evaluating changes in transcript levels, it is possible to understand the basic mechanisms of intercellular communication, and the intracellular cascades that underlie a gene's biological role in encoding a protein.

Additionally, changes in expression patterns of multiple genes may be informative for understanding regulatory mechanisms, biochemical pathways, or cellular functions (Lockhart and Winzeler, 2000; Napoli et al., 2005). In the future, gene expression patterns are expected to be helpful in identifying the causes and consequences of disease, determining the efficiency of drugs and drug candidates in cells and organisms, and for recognizing gene products with therapeutic value or as targets for therapeutic intervention (Lockhart and Winzeler, 2000). Therefore, accurately evaluating changes of gene expression is now critical for many diseases.

There are several methods for measuring gene expression. In terms of the number of genes detected at a time, tools for measuring gene expression can be divided into two categories: single- or multiple-genes analyses at a time (Lockhart and Winzeler, 2000; Gibson and Muse, 2002). Each method has its advantages and disadvantages. The choice of a method is influenced by considerations including labor, economic, and the aims of the experiment.

Methods for measuring thousands of differentially expressed genes include expressed sequence tags (ESTs) (Adams et al., 1991), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), and microarrays (Gibson and Muse, 2002). Here, I will show their advantages and disadvantages with examples about cardiac studies.

Expressed sequence tags are short sequences of cDNAs generated by

single-pass sequencing of either one or both ends of expressed genes from certain tissues, cells, or organs. Tags generated from both normal and abnormal tissues can be used to identify differently expressed genes (Adams et al., 1991). To date, ESTs from many species are available in public databases including chicken, pig, and cattle (<http://www.ncbi.nlm.nih.gov>). Through sequencing analysis and BLAST, ESTs can also be used to catalogue the identities and expression levels of genes, and to discover novel genes. Hwang et al. (1997) used sequencing analysis and BLAST to catalogue 84,904 ESTs from 13 independent cardiovascular system-based cDNA libraries. 12% of them were possibly novel genes in cardiovascular system. ESTs matched to known genes were classified according to function and allowed for detection of differences in transcription patterns. They also identified 48 genes potentially over-expressed in cardiac hypertrophy, at least 10 of which were documented as differentially expressed. These ESTs are thus important sources for studying complex diseases including DCM using microarrays (Barrans et al., 2002). However, the primary limitation of ESTs is the requirement for high-throughput DNA sequencing capabilities and expertise in bioinformatic analysis of data set (Moody, 2001).

Serial analysis of gene expression first described by Velculescu et al. (1995), SAGE has also been used to evaluate differences in gene expression. Principles of SAGE are based on two steps: 1) Short sequences are generated that can effectively

identify the original transcripts; 2) linking these tags allows for rapid sequence analysis of multiple transcripts. The advantages of SAGE include a 30-50 fold more information per sequencing reaction when compared to ESTs; more effective than ESTs for identifying rare transcripts and more reliable quantitative data. Because of these advantages, this method has been gradually used for discovering novel targets, including characterized genes of known function, annotated genes of unknown function, and the putative genes not yet present in current databases. Schwartz et al. (2004) used SAGE to study the regulation of ANG II, an oligopeptide, which causes multifocal myocardial lesions which lead to cardiac hypertrophy and heart failure, in the mouse. Several novel genes were identified and distinct expression profiles showed that four of them were in response of ANG II. Functions of six genes remained unknown may be associated with the regulation. The other genes were thus characterized to involve in different functions during the regulation of ANG II. In terms of characterization, for example, phospholamban and calreticulin genes were down-regulated and were characterized to affect intracellular calcium homeostasis. Genes encoding three LIM-domain proteins, Csrp1, Csrp3, and Crip1, known to interact with the filamentous actin, were up-regulated. Disrupted *Csrp3* gene has been reported to develop DCM with hypertrophy and heart failure.

The limitations of SAGE include the need for extensive sequencing and tags

can only be used to identify genes where sequences are in databases (Moody, 2001).

Additionally, since SAGE is new, its application in livestock and poultry has been limited.

Another method of choice for the expression of thousands of genes is microarrays. Gene expression using microarray technology was originally described by Schena et al. (1995). The basic concept involves parallel hybridization of probes to targets on glass slides. There are two types of microarrays: oligonucleotide and cDNA. Oligonucleotides are synthesized *in situ* on the glass slide whereas cloned cDNAs are deposited or sported on the glass slide (Lockhart and Winzeler, 2000; Gibson and Muse, 2002). The advantages of microarrays include extraordinarily high throughput and sensitivity. But, they are limited by the need for sophisticated software and expertise to analyze generated data (Moody, 2001).

In spite of some disadvantages, microarrays are still considered powerful tools for scanning thousands of genes, suspected to be associated with complex diseases. Recently, microarray analysis was used to gain more understanding about cardiovascular diseases, including DCM. Tan et al. (2002) used oligonucleotide microarrays to profile non-failing and failing human hearts diagnosed with end-stage DCM. A total of 19 genes were reported to be differentially expressed. Surprisingly, these genes did not include *cTnT* and *PLN*. There was probably because those patients

suffered from end-stage of DCM and had been treated with medications, such as epinephrine. Although this study may be helpful for understanding gene expression profiles, it also indicates that studying DCM using human model may not be convenient.

Commonly used measurement to alternative whether only a few genes are expressed in samples include *in situ* hybridization, Northern blot, and reverse transcription polymerase chain reaction (RT-PCR) (Bustin, 2000; Gibson and Muse, 2002). Northern blots are semi-quantitative and the simplest procedure (Gibson and Muse, 2002). If transcripts of interest are alternatively spliced, northern blots can identify this phenomenon. Additionally, northern blots can provide the information about the size of mRNA and the integrity of RNA samples (Bustin, 2000). Though northern blots are limited by detecting two fold or greater differences in gene expression, it is still useful for measuring changes less than two fold. A study of oxidative stress associated with the end-stage of heart failure conduced by Dieterich et al (200) used Northern blot analysis to measure expression of 4 candidate genes. These candidate genes encode manganese superoxide dismutase, copper-zinc superoxide dismutase, glutathione peroxidase, and catalase are scavengers for the cytotoxic effects of reactive oxygen metabolites, which is involved in the pathogenesis as well as the progression of heart failure. Changes of these four gene expressions were less then two

fold and only catalase gene significantly differentially expressed between no-failure and failure hearts caused by DCM or ischemic cardiomyopathy. The up-regulated catalase gene expression may be result from increased oxidative stress in human end-stage heart failure.

The approach of *in situ* hybridization is similar to Northern blot except that the fixed tissue is probed on the solid medium. *In situ* hybridization can localize transcripts to specific cells within tissues. Stabej et al. (2005) studied the characterization of the canine sarcoglycan delta (*SGCD*), a gene implicated in DCM in human and hamster. Though they found no association between any of variants of dogs, fluorescent *in situ* hybridization allowed them to localize this gene on chromosome 4q22. But this method is laborious, time consuming, and not quantitative. In addition, *in situ hybridization* is more complex than other expression methods (Pauschinger et al., 2004; Bustin, 2000).

The main limitation of northern blots and *in situ hybridization* methods is that they are less sensitive than RT-PCR. RT-PCR, which was also used in this project, is the most sensitive and flexible method for quantification of transcripts. It can be used to compare gene differentially expressed in different sample populations, and to analyze RNA structure, and to identify closely related mRNA expression. This method is the primary choice for mutation analysis and expression analysis due to its fidelity (Bustin,

2000), and it is also an important tool for the validation of microarrays and SAGE experiments (Cook et al., 2004; Schwartz et al., 2004). Because of the great advantages of RT-PCR, it has been utilized to diagnosis of therapeutic efficiency and diseases. Shigeyama et al. (2005) used RT-PCR to examine expression levels of three cardiac candidate genes in DCM affected patients treated with beta-blockers, a drug inhibiting the growth of blood vessels. These three genes included collagen Type I (Col I) and Type III (Col III), important genes in myocardial contractile function and cardiac remodeling, and transforming growth factor- β_1 (TGF- β_1), an important indicator of gene expression of Col I and Col III. The expression levels of Col I and Col III were examined to be reduced after the beta-blocker treatment. Clinical diagnosis showed significant improvement in symptoms of left ventricular end-diastolic volume, and ejection fraction, the plasma concentration of brain natriuretic peptide, and cardiac sympathetic nerve activity. These results indicate that RT-PCR method is useful for diagnosis of therapeutic efficiency.

For diagnosis of diseases, RT-PCR has been useful for detecting patients with enteroviruses, important factors for the development of myocarditis. Pauschinger et al. (1999) used RT-PCR in conjunction with Southern blot hybridization to detect enteroviral RNA in 45 patients with left ventricular dysfunction and ejection fraction. Forty percentages of patients were detected to carry enteroviral RNA, which were not

found at all in the control group. A study of 26 DCM patients conducted by Fujioka et al. (2000) showed that coxsackie B viruses, such as coxsackievirus B3 and B4, were the most seen in hearts with IDCM patients among eight kinds of viruses using RT-PCR and PCR. This study indicates that RT-PCR can also be used to study the association between diseases and pathogens.

2.4 Single nucleotide polymorphism

Single nucleotide polymorphisms (SNPs) are DNA sequence variants that involve nucleotide changes such as single-base insertions and deletions (Gibson and Muse, 2002). In a given population, SNPs are nucleotide variants with a frequency greater than one percent. It is estimated that SNPs occur approximately every 500-1,000 bases in the human genome (Riva and Kohane, 2002).

If a SNP has functional consequences such as altering the expression of a gene, it may contribute to development of disease (Gibbons et al., 2004). For example, Venkatraman et al. (2005) identified a point mutation in the turkey that affects the sensitivity of troponin complex to Ca^{2+} and leads to impaired systolic function and cardiac dilation in the turkey. In human, a similar point mutation (C to T) located at nucleotide 471 of the *cTnT* gene (GenBank Accession No. NM_000364) was identified in a family with familial DCM. This mutation determines the phenotype of DCM (Li et al., 2001). On the other hand, even if a SNP has no functional consequences, it may be

close to a functional variant such that both the SNP and the variant tend to be inherited together or in linkage disequilibrium. This latter type of SNP is useful for disease prediction as well as for the eventual isolation of functional variants (Gibbons et al., 2004).

Single nucleotide polymorphisms are also informative for studying the evolution of species, and the history and mutual relationships of different human populations (Riva and Kohane, 2002). In addition to their use in human biology and evolution, SNPs have also been used in animal studies to evaluate the relationships among species including turkey strains (Smith et al., 2005). Among the SNPs, detected was one that seemed to be strain-specific for which a restriction fragment length polymorphism genotyping method was developed. Their overall results suggested that two turkey strains are genetically related to each other.

It is also believed that SNPs are valuable sources of genetic markers for constructing genomic maps and predicting disease risk (Suh and Vijg, 2005). Yamada et al. (2002), for instance, investigated genotyped 112 polymorphisms in 71 candidate genes associated with myocardial infarction in 2,819 unrelated Japanese patients and 2,242 unrelated Japanese controls. Variants in 3 genes including connexin 37, plasminogen-activator inhibitor 1, and stromelysin-1 were associated with increased risk of myocardial infarction. This study indicates that predicting the risk of a complex

disease is possible and will be helpful for development of preventive therapy ahead in the future.

Single nucleotide polymorphisms have also been used to identify the genetic differences associated with individual response to drugs (Lee et al., 2005). Perez et al. (2003) examined the responses of patients polymorphic for residue 389 (Arg/Gly polymorphism) in β_1 -adrenergic receptor (β_1 -ARs) to Carvedilol, a beta blocker that reduces the progression of heart failure. Patients with homozygous Arg389 variant had greater improvement in ventricular function compared with Gly389-homozygous patients. Heterozygous patients showed similar improvement to that of Arg389-homozygous patients. This study successfully not only shows another utility of SNPs, but also indicates that genetic variants affected drug efficacy is allowed to be detected before the therapies.

Based on the SNPs information, SNP map is able to be established. For example, a map of human genome sequence variation containing 1.42 million SNPs was established by the International SNP Map Working Group (2001). This map provides an average density on available sequence of one SNP every 1.9 kilobases and has about 60,000 SNPs within exon. Additionally, about 85% of exons are within 5 kb of the nearest SNP. This high density SNP map is expected to provide a public resource for studying human haplotype structure, which is set of closely linked alleles, and for

helping identify biometrically important genes for diagnosis and therapy.

At present, there are several high-throughput systems available for rapid SNP genotyping including TaqMan assay, BeadArray, and SNP-IT tag array. The capability of genotyping and available SNP map appear to be surpassing that for linkage studies (Niu et al., 2002; The International SNP Map Working Group, 2001). In many cases, a single SNP provides relatively low information for a genotype due to weak linkage disequilibrium (LD) signal. Especially, a genotype of a complex disease that multiple genes involved in pathogenesis is interested (Niu et al., 2002). When one SNP is used to approach with multiple candidate genes, tens of thousands of samples are required for having sufficient power to detect significant associations. Therefore, the haplotype approach will be also helpful to increase popularity and informative for predicting prognosis of certain genetic diseases and helping map the locus of markers (Olson and Keating, 1996; Lee et al., 2005).

CHAPTER 3

Expression analysis of candidate genes for toxin-induced dilated cardiomyopathy in the turkey (*Meleagris gallopav*)

3.1 ABSTRACT

Though several studies have previously identified some genes that influence idiopathic DCM, none has been described for toxin-induced dilated cardiomyopathy. Here, we compared the expression levels of two candidate genes including cardiac troponin T (cTnT) and phospholamban (PLN) in turkeys induced by Furazolidone to have dilated cardiomyopathy. Primers were designed from homologous and heterologous gene sequences for cTnT and PLN, respectively. A total of 18 birds, 11 DCM-affected and 7 unaffected, were used in the quantitative reverse transcription polymerase chain reaction (RT-PCR). Differences between affected and unaffected turkeys in the expression of both cTnT and PLN in heart and liver were not significant. The sequence of the turkey PLN showed significant similarity at the nucleotide and amino acid levels to the reference chicken sequence and those of other species. Though the present work did not show any significant association between DCM and expression levels of cTnT and PLN, it led to the development of genomic reagents including the cDNA sequence and the primers for RT-PCR for PLN that could be useful for future association studies.

Keywords: Turkey, cardiomyopathy, Cardiac troponin T, Phospholamban, RT-PCR

3.2 Introduction

Idiopathic dilated cardiomyopathy (IDCM), a disease that affects many species, has been attributed to mutations in genes that influence the cytoskeleton system. Recently, Gyenai (2005) showed that toxin-induced DCM is also genetic. In a comparison of five turkey varieties, significant differences were observed in the incidence and severity of DCM. Candidate genes respectively for these differences may be similar to those that influence IDCM, because IDCM and toxin-induced DCM are reported to be similar in physiological and biochemical characteristics.

Measurements of gene expression have the advantage of providing biological understanding of phenotypes and phenomena, such as the cause and consequences of diseases (Napoli et al., 2005). In human diseases and those of other animals, gene expression profiling studies have allowed better understanding of pathophysiological processes. Additionally, they may lead to the development of new clinical tools to improve diagnosis and prognosis of specific diseases based on different expression patterns (Steenman et al., 2005).

In the first genome-wide expression profile analysis of human IDCM, Crzeskowiak et al. (2003) observed that 364 out of 30,336 cDNA clones were differentially expressed. Thirty out of 220 of the regulated transcripts were found to be involved in heart contractility and function including cardiac troponin T (cTnT) and

phospholamban (PLN). Other studies conducted by Schwinger et al. (1995) and Biesiadecki and Jin (2002) have shown that differentially spontaneous expression in PLN and cTnT genes, respectively.

The PLN gene encodes phospholamban protein, an important regulator of sarcoplasmic reticulum (SR) calcium uptake. If a reduced Ca^{2+} loading of the SR is present in failing human myocardium, then diastolic relaxation and the frequency-dependent increase in force development will be altered. A reduced SR Ca^{2+} uptake may also lead to a slower diastolic Ca^{2+} decay and a reduced SR Ca^{2+} content, which will cause less available Ca^{2+} content to be released during depolarization (Movesesian et al., 1989). Reduced expression of PLN at RNA level has been observed in animal models of cardiac dysfunction, and may contribute to diastolic dysfunction in failing hearts. Though reports about this gene are negligible in the turkey, PLN has been studied in different species, including mice, human, and chicken. Additionally, regions of the PLN gene have been shown to be conserved in many species (Koss and Kranias, 1996). This conservation has made it easier to clone the PLN and characterize it in many species. For example, recently Stabej et al. (2005) used this conservation to characterize PLN in dogs affected by DCM.

Cardiac TnT encodes a protein, which is central subunit of the troponin complex in the thin filament. This protein plays an important role in the sensitivity of

the myofilaments to Ca^{2+} during striated muscle contraction. Abnormalities of this protein caused by mutations disrupt the Ca^{2+} kinetics in the cell, thus causing myopathy.

Biesiadecki and Jin (2002) reported that an unusual low molecular weight cTnT was expressed in inherited DCM turkey and failing hearts due to a mutation that causes aberrant splicing.

Reverse transcription PCR has become increasingly important in a large number of clinical and scientific fields for the evaluation of the effect of candidate genes due to its sensitivity and accuracy in expression analyses. Because of the increased use of RT-PCR in gene analysis, the techniques continue to be required to further increase sensitivity and accuracy. For example, to compensate for differences in the purity and concentration of the samples introduced during sample preparation normalization is carried out. As in most analyses, these differences can be equalized by normalization to an internal standard, such as endogenous reference or housekeeping genes (Gilsbach et al., 2006).

Since others have previously showed that PLN and cTnT expression are affected in spontaneous DCM, here I used RT-PCR to evaluate the variation in expression of these candidate genes in toxin-induced DCM in the turkey. A secondary objective was to isolate a partial turkey PLN cDNA sequence if it is currently not available in public data bases.

3.3 Materials and methods

3.3a Animals and tissue collection

Crossbred turkeys used in the present work were from matings of Bourbon Red (BR), Spanish Black (SB), and Royal Palm (RP). A total of 18 birds, including 12 BR/N and 6 RP/N crosses, were used. The rationale for using crosses was to increase the likelihood of a uniform genetic background. The birds were randomly assigned to treatment (n=11) and control (n=7) groups from hatch. Both groups of poulets were fed *ad libitum*. The birds in the treatment group were fed with a standard turkey poult diet containing 700 parts per million of furazolidone (Fz) as described by Czarnecki (1984) and Gjenai (2005).

Birds were scanned each week for DCM using Echocardiography (ECHO Akola) as described by Gjenai (2005). Birds were selected for tissue collection for the expression studies based on left ventricular end-diastolic (LVEDD) and left ventricular end-systolic dimensions (LVESD) determined from the ECHO. These parameters have previously been shown to be consistent indicators of DCM (Gjenai, 2005). Both heart and liver from control and treatment birds were collected at 7 and 14 days-of-age. Once the tissues were collected, they were washed in phosphate buffered saline and quickly frozen in liquid nitrogen followed by storage at -80°C before using for RNA isolation.

3.3b Total RNA isolation

In preliminary investigations, blood was evaluated as a source of total

RNA for the expression analysis. Blood was collected by brachial venipuncture in tubes containing 0.5M EDTA. RNA was extracted according to the manufacturers recommended protocol (Gentra System Inc., Minneapolis, MN). The concentration and quality of each sample were determined by standard protocols (Sambrook and Russell, 2001). The quality was further verified by electrophoresis on formaldehyde-agarose gels.

Total RNA from both heart and liver tissue samples was extracted by using the RNeasy Midi Kit according to the manufacturers recommended protocol (Qiagen Inc., Valencia, CA). The RNA concentrations were determined by Virginia Bioinformatics Institute (VBI) using the Agilent BioAnalyzer 2100 and RNA quality was verified by electrophoresis on 1 % formaldehyde-agarose gels. RNA samples were treated with DNase to remove away genomic DNA (Qiagen Inc., Valencia, CA).

3.3c Primer design and optimization

Primers were designed using the Primer 3 program (Rozen and Skaletsky, 1997). The turkey cTnT and chicken PLN mRNA GenBank sequences of Accession No. AF005139 and NM_205410, respectively, were used in the primer design. Prior to primer design, the sequences were determined to be conserved in a Clustal-W-based multiple sequence comparison. Beta-actin primers were designed using GenBank sequence, Accession No. NM_205518.

Primers were optimized using the FailSafe PCR PreMix Selection kit (Epicentre Inc., Madison, WI). The optimization PCR was carried out in a total volume of 25 µl containing 100 ng of genomic DNA, 50 pmol of each primer, 12.5 µl of FailSafe PCR 2X Pre Mix, and 1.25 units of FailSafe PCR Enzyme Mix. The optimization cycling and times were as follows: denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 45 s, appropriate annealing temperature in Table 3.1 for 45 s, and 70°C for 45s. PCR products were analyzed on a 2% agarose gel and showed with SYBR green.

3.3d Reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis

The RT-PCR was carried out at the Core facilities of the VBI (Virginia Bioinformatics Institute, Blacksburg, VA). Total RNA (1µg) was transcribed to cDNA using the Biorad I-script cDNA synthesis kit (Biohad, Hercules, CA) in a total volume of 20 µl. Negative controls were processed with the samples to test for non-specific transcription or amplification. Each cDNA was diluted 1:10 with RT-grade PCR water (Ambion, Austin, TX). Reactions consisted of 300 nM sense and anti-sense primers, 1µl diluted cDNA, and RT-grade PCR water to obtain a volume of 12.5 µl. SyBr-Green Supermix (Biorad, Hercules, CA) was added to obtain a final reaction volume of 25 µl. The PCR amplification was initiated by heating at 95°C for 3 min, followed by 40 cycles of the following conditions: 10s at 95°C, 15 s at annealing temperature (Table

3.1), 20 s at 72°C. Products from RT-PCR were analyzed on a 2% agarose gel containing ethidium bromide.

To validate the RT-PCR, the cDNAs were sequenced using standard BigDye termination procedure at Core facilities of the VBI. The nucleotide sequences were compared to database sequences using BLAST. An extra base of thymine (T), which exists in chicken phospholamban (PLN) sequence, but not in the PLN cDNA sequence of this project, was inserted in the 121st base to make our PLN cDNA sequence be able to be divided by 3 with no remainder. Using Open Reading Frame Finder program, the sequence was translated (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). This translated protein was compared to other protein sequences of other species using BLAST program.

3.3e Statistical analyses

The gene expression data were normalized to a reference gene (β -actin) and calibrated to the control group by $2^{-\Delta C_T}$ method (Livak and Schmittgen, 2001), where $C_T = (C_{T, \text{Target}} - C_{T, \beta\text{-actin}}) - (C_{T, \text{Calibrator}} - C_{T, \beta\text{-actin}})$. The converted data were log-transformed to fit normal and then, analyzed using the MIXED-model procedure of the Statistical Analysis System (SAS Institute, 2003). The model included Age, Group, and Cross as main effects and test of appropriate two-way interactions. Differences were considered significant if $P < 0.05$.

3.4 Results and Discussion

In the preliminary analysis, RNA isolated from blood was limited in amount and quality. RNA isolated from the blood is presented in Figure 3.1. The concentration of the RNA was significantly below that needed for RT-PCR (data not presented). Because of this low concentration, multiple expression was not determined from the same bird as was anticipated.

The sizes of all RT-PCR products were close to the expected (Figure 3.2). The BLAST result showed that the turkey β -actin cDNA sequence had 99 % similarity to turkey β -actin mRNA sequence in GenBank (Figure 3.3). Cardiac troponin T cDNA sequence also showed a 99% nucleotide sequence similarity to GenBank turkey cTnT mRNA sequence (Figure 3.4). The 278-bp turkey PLN cDNA sequence showed 98% similarity to chicken PLN cDNA sequence (Figure 3.5) and at least 81% to the PLN mRNA sequence of other species (Table 3.2). The translated product of the turkey PLN sequence showed a region of strong conservation with other species including pig, dog, human, chicken, and mouse (Figure 3.6).

The ECHO and relative gene expression data in both heart and liver tissues from normal and Fz-induced birds in the three crosses evaluated are shown in Tables 3.3, 3.4, 3.5, and 3.6. The birds on Fz-containing diet had significantly larger LVEDD and LVESD (Table 3.3). The dilation of the left ventricles of birds on Fz-containing

diet is consistent with previous reports (Czarnecki et al., 1973; Enzig et al 1981; Gyenai et al., 2005).

Statistical analysis of the gene expression data showed that Age x Group interaction PLN and cTnT appeared to be significant ($P < 0.05$) in heart. The expression of cTnT and PLN in 14 day-old birds increased about 267% and 231%, respectively, over that in 7 day-old birds on Fz-diet.

Expression levels of cTnT and PLN in heart and liver of normal and Fz-induced birds are show in Figure 3.7 and 3.8, respectively. The differences between control and treatment birds in mRNA levels of PLN and cTnT were not significant. Expression level of cTnT in birds in the treatment group decreased about 61% in heart. Gene expression level of PLN increased 18% in Fz-induced birds relative to normal birds in heart. In liver, cTnT and PLN mRNA levels increased about 110 and 213 %, respectively, in treatment group relative to control group.

The lack of a significant association of PLN expression with IDCM is inconsistent with studies involving IDCM. For example, overexpression of cardiac PLN in transgenic mice has been shown to lead to a late-onset type of cardiomyopathy (Dash et al., 2001). Additionally, transgenic mice with high PLN level (400%) compared with wild type of mice produced a greater inhibition of calcium kinetics and showed morphological alterations in heart. In human, mRNA expression level of PLN

decreased significantly (67%) in failing heart caused by idiopathic DCM. In affected individuals, low levels of PLN mRNA were observed in smooth muscle organs and little or no expression in non-muscle organs (Schwinger et al., 1995). In a study by Toyofuku and Zak (1991), PLN transcripts were found to be expressed only in cardiac and slow-tonic muscle during development in 7 day embryo, 15 day fetus, neonate through adult, but not in liver, brain, or gizzard of normal chickens.

Like PLN expression, cTnT expression has also been shown to be altered in same IDCM-affected animals. Biesiadecki and Jin (2002) reported an abnormal splicing of exon 8 in cTnT of turkeys with inherited DCM. The exclusion of exon 8 results in significant changes in the conformation of cTnT, which alters interactions with other myofilaments and Ca^{2+} sensitivity of myosin ATPase activity. In idiopathic DCM patients, cTnT mRNA level has also been observed to be in a similar degree of deregulation (Grzeskowiak et al., 2003). These different results may be due to different genetic mechanisms.

There are several reasons why the differences between DCM-affected and normal birds were not significant. It is possible that idiopathic DCM, and not Fz-DCM is affected by differential expression of cTnT and PLN. It may also be that species differences in association with DCM exist for both cTnT and PLN. For example, PLN expression in six DCM-affected dogs remained unaffected (Stabej et al., 2005).

The selected reference gene (β -actin) has previously been described as a candidate gene for DCM because cytoskeleton proteins play important roles in maintaining cell structure. For example, defects in α -actin can damage cell strength and may cause cardiomyopathy (Maeda et al., 1997). Beta-cardiac actin, which is a cytoskeleton protein, has been reported to be up-regulated in end-stage human DCM (Barrans et al., 2002). In the present work, the β -actin expression was a control, which did not appear to be different in both treatment and control birds. In the future, additional reference genes may be needed to help validate a conclusion of no differential change in β -actin as observed here (Gilsbach et al., 2006).

Greater differential expression of both genes was detected in liver. However, this great differential expression was statistically proved not significant and had great values in standard error. This may be caused by the oversensitivity of RT-PCR, a smaller sample size, or individuals responded to Fz differently.

In summary, expression levels of PLN and cTnT genes in Fz-fed turkey were first time studied. Our results currently suggest that expression of PLN and cTnT genes were not associated with toxin-induced DCM in the turkey. These expression results and the reagents produced in this study may be informative for microarray study in the future.

Table 1. Sequences of primers used in the real-time polymerase chain reaction

Accession number	Gene name	Primers [†]	Amplicon length	Tm* (°C)
AY005139	cTnT	Forward : 5' AAAATCCCCGATGGTGAG 3' Reverse: 5' GCTCAATCCTGTCTTGAGG 3'	154	56
NM_205410	PLN	Forward: 5' GAGGAGAGCCTCAACTCTG 3' Reverse: 5' ATACATGTGGCAGGCAGTAA 3'	292	58.2
NM_205518	β-actin	Forward: 5' TGATGGTGTACCCACACTG 3' Reverse: 5' TTCTCCAGGGAAGAGCTAGA 3'	246	56

[†]The primers were derived from turkey cardiac troponin T (cTnT) and chicken phospholamban (PLN) and β-actin sequences in GenBank.

*Tm represents the optimized annealing temperature at which a single amplicon of the expected size was obtained.

Table 2. Multi-sequences comparison of turkey phospholamban cDNA sequence

Species	Identities [†] (%)	Gaps (bp)	Accession number*
Chicken	241/245 (98%)	2/245 (0%)	NM_205410.1
Human	85/99 (85%)	0/99 (0%)	NM_002667.2
Pig	58/65 (89%)	0/65 (0%)	X15075.1
Dog	57/65 (87%)	0/65 (0%)	Y00399.1
Rabbit	81/99 (81%)	0/99 (0%)	M63601.1
Rat	59/71 (83%)	0/71 (0%)	L03382.1

* Accession numbers of matched sequences in GenBank.

† The length of the GenBank turkey sequence in the BLAST-2 alignment.

Table 3. Mean and standard error of echocardiographic (ECHO) and cardiac troponin T (cTnT) mRNA expression (arbitrary units as a ratio: cTnT/β-actin)*

	SB/BR				BR/N				N/BR	
	ECHO		ECHO		ECHO		ECHO		mRNA expression	
	LVEDD [†]	LVESD [†]	mRNA expression		LVEDD	LVESD	LVEDD	LVESD		
TRT	--	--	0.0667 ± 0.4452	1.12 ± 0.41 ^{b1}	1.05 ± 0.3 ^{b1}	-0.00175 ± 0.2548	0.72 ± 0.32 ^{b3}	0.74 ± 0.21 ^{b2}	0.2807 ± 0.2548	
CTL	0.47 ± 0.06 ¹	0.31 ± 0.05 ²	0.0134 ± 0.1963	0.47 ± 0.14 ^{a1}	0.49 ± 0.16 ^{a1}	0.06124 ± 0.3604	0.43 ± 0.14 ^{a1}	0.42 ± 0.11 ^{a12}	0.9450 ± 0.3604	
P value	--		0.9181		< 0.05		0.8921		< 0.05	0.1926

* SB/BR, BR/N, and N/BR represent Spanish Black/ Bourbon Red, Bourbon Red/Narragansett, and Narragansett/Bourbon Red, respectively. TRT and CTL represent expression in heart obtained from birds on Fz-containing and normal diet, respectively. Arbitrary unit represents log-transformed fold.

[†]LVEDD and LVESD are left ventricular end-diastolic dimension and left ventricular end-systolic dimension, respectively, as determined from ultrasound measurements by echocardiography.

^{a,b}Measurements in the same column with similar alphabetic superscript are not different ($P>0.05$).

^{1,2}Values in the same row with similar numeric superscript are not significantly ($P>0.05$).

(ECHO data provided by Kwaku B. Gyenai, 2005)

Table 4. Mean and standard error of echocardiographic (ECHO) and phospholamban (PLN) mRNA expression (arbitrary units as a ratio: PLN/ β -actin)*

Group	SB/BR			BR/N			N/BR		
	ECHO		mRNA expression	ECHO		mRNA expression	ECHO		mRNA expression
	LVEDD [†]	LVESD [†]		LVEDD	LVESD		LVEDD	LVESD	
TRT	--	--	2.59 ± 0.894	1.12 ± 0.41 ^{b1}	1.05 ± 0.3 ^{b1}	-0.077 ± 0.1766	0.72 ± 0.32 ^{b3}	0.74 ± 0.21 ^{b2}	0.2051 ± 0.1766
CTL	0.47 ± 0.06 ¹	0.31 ± 0.05 ²	1.2133 ± 0.8335	0.47 ± 0.14 ^{a1}	0.49 ± 0.16 ^{a1}	0.2276 ± 0.2498	0.43 ± 0.14 ^{a1}	0.42 ± 0.11 ^{a12}	0.7036 ± 0.2498
P value	--		0.3092		< 0.05	0.3652		< 0.05	0.1692

*SB/BR, BR/N, and N/BR represent Spanish Black/ Bourbon Red, Bourbon Red/Narragansett, and Narragansett/Bourbon Red, respectively. TRT and CTL represent expression in heart obtained from birds on Fz-containing and normal diet, respectively. Arbitrary unit represents log-transformed fold.

[†]LVEDD and LVESD are left ventricular end-diastolic dimension and left ventricular end-systolic dimension, respectively, as determined from ultrasound measurements by echocardiography.

^{a,b}Measurements in the same column with similar alphabetic superscript are not different ($P>0.05$).

^{1,2}Values in the same row with similar numeric superscript are not significantly ($P>0.05$).

(ECHO data provided by Kwaku B. Gyenai, 2005)

Table 5. Mean and standard error of cardiac troponin T (cTnT) mRNA expression
(arbitrary units as a ratio: cTnT/ β -actin)*

	SB/BR*	BR/N*	N/BR*
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
TRT	3.0433 \pm 0.2791	0.3838 \pm 0.3713	-1.0028 \pm 0.3713
CTL	3.9267 \pm 2.3735	-0.3859 \pm 0.5252	-0.3535 \pm 0.5252
P value	0.7304	0.2851	0.3591

* SB/BR, BR/N, and N/BR represent Spanish Black/ Bourbon Red, Bourbon Red/Narragansett, and Narragansett/Bourbon Red, respectively. TRT and CTL represent expression in liver obtained from birds on Fz-containing and normal diet, respectively. Arbitrary unit represents log-transformed fold.

Table 6. Mean and standard error of phospholamban (PLN) mRNA expression
 (arbitrary units as a ration: PLN/ β -actin)*

	SB/BR*	BR/N*	N/BR*
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
TRT	-1.837 \pm 0.742	1.2153 \pm 0.4821	0.7186 \pm 0.4821
CTL	-1.677 \pm 1.131	0.3951 \pm 0.6818	0.2449 \pm 0.6818
P value	0.9116	0.3711	0.5951

* SB/BR, BR/N, and N/BR represent Spanish Black/ Bourbon Red, Bourbon Red/Narragansett, and Narragansett/Bourbon Red, respectively. TRT and CTL represent expression in liver obtained from birds on Fz-containing and normal diet, respectively. Arbitrary unit represents log-transformed fold.

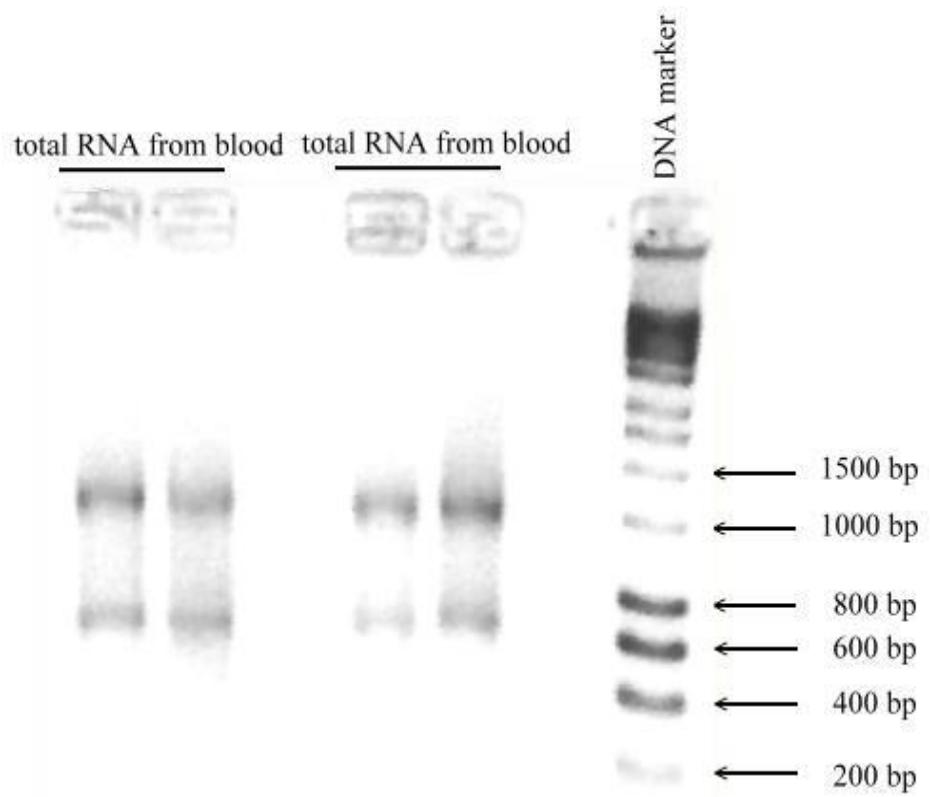


Figure 1. Formaldehyde agarose gel patterns of RNA isolated from turkey blood.

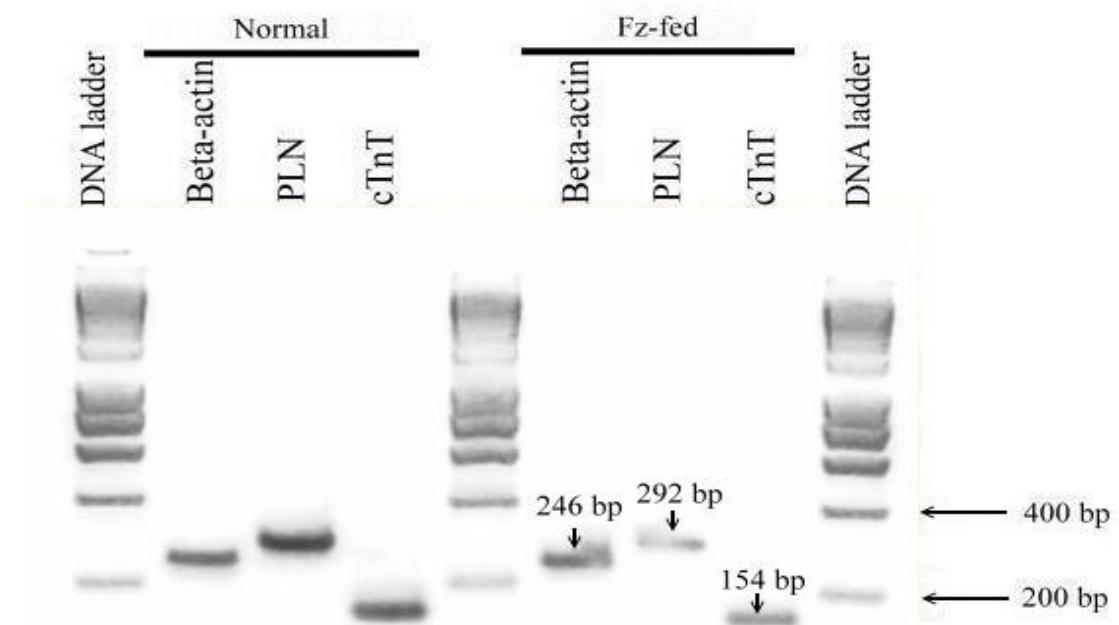


Figure 2. Agarose gel patterns of turkey amplicons produced by primers-specific for turkey cardiac troponin T, chicken phospholamban, and chicken beta-actin. Based on the predicted primer binding sites in the turkey and chicken, the expected RT-PCR product sizes were 154, 292, and 246 bp, respectively. These amplicons were amplified from 7-day-old birds.

Query Sbjct	1 527	GCTATGCCCTCCCC-ATGCCATCCTCCGTCTGGATCTGGCTGCCGTGACCTGACGGACT GCTATGCCCTCCCCCATGCCATCCTCCGTCTGGATCTGGCTGCCGTGACCTGACGGACT	59 586
Query Sbjct	60 587	ACCTCATGAAGATCCTGACAGAGAGAGGGCTACAGCTTCACTACCACAGCCGAGAGAGAAA ACCTCATGAAGATCCTGACAGAGAGAGGGCTACAGCTTCACTACCACAGCCGAGAGAGAAA	119 646
Query Sbjct	120 647	TTGTGCGTGACATCAAGGAGAAGCTGTGCTACGTCGCACTGGATT TTGTGCGTGACATCAAGGAGAAGCTGTGCTACGTCGCACTGGATT	164 691

Figure 3. BLAST-2-alignment of the turkey cDNA sequence of the amplicon produced using primers-specific for chicken β -actin mRNA, Accession No. AY942620, with GenBank turkey β -actin mRNA sequence, Accession No. NM_205518. The almost perfect match provides a strong evidence of the validity of the gene expression analysis.

Query	115	GACATCCACCGCAAGCGCATGGAGAAGGACCTGAATGAGCTGCAGGCCCTCATCGAACCC	174
Sbjct	280	GACATCCACCGCAAGCGCATGGAGAAGGACCTGAATGAGCTGCAGGCCCTCATCGAACCC	339
Query	175	CATTTGAGAGCAGGAAGAAGGAGGAAGAAGAGCTCATCTCCCTCAAAGA-AGGATTGAG	233
Sbjct	340	CATTTGAGAGCAGGAAGAAGGAGGAAGAAGAGCTCATCTCCCTCAAAGACAGGATTGAG	399
Query	234	CA 235	
Sbjct	400	CA 401	

Figure 4. BLAST-2-alignment of the turkey cDNA sequence of the amplicon produced using primers-specific for turkey cardiac troponin T (cTnT), Accession No. AY005139, with GenBank turkey cTnT mRNA sequence. The almost perfect match provides a strong evidence of the validity of the gene expression analysis.

TK PLN	1	GGCAGTAAGAGCAGCGCATGGCTGGCACACAGCACAGCCTAGTCCAAGTGCTAGCCCC	60
CK PLN	560	GGCAGTAAGAGCAGCGCATGGCTGGCACACAGCACAGCCTAGTCCAAGTGCTAGCCCC	501
TK PLN	61	TCTCCAAGGGATCCTGCATTGGGTTGGAAGTGGAGGTCGCTTCCCTTGGCGG	120
CK PLN	500	TCTCCAAGGGATCCTGCATTGGGTTGGAAGTGGAGGTCGCTTCCCTTGGAGG	441
TK PLN	121	CTTCCTAGAGAACAGTCAGAGAAGTGCTGGAACCTCAGAGGAGCATACAATGATACA	180
CK PLN	440	CTTCCTAGAGAACAGTCAGAGAAGTGCTGGAACCTCAGAGGAGCATACAATGATACA	381
TK PLN	181	GATCAGCAAGAGGCCAAATTAAAGATCAGGCAGAAA-TCACAAAGAGCTCCTGGAGCCTTG	239
CK PLN	380	GATCAGCAAGAGGCCAAATTAAAGATCAGGCAGAAATTACACAAAGAGCTCCTGGAGCCTTG	321
TK PLN	240	GCGTGCTTGTGGGTTGACCTCAAGAGTTGAGGCTCTCCT	278
CK PLN	320	GCGTGCTTGTGGGTTGACCTCAAGAGTTGAGGCTCTCCT	282

Figure 5. BLAST-2-alignment of the turkey cDNA sequence (TK PLN) of the amplicon produced using primers-specific for chicken phospholamban (PLN) mRNA sequence, Accession No. NM_205410, with GenBank chicken PLN mRNA sequence (CK PLN). The almost perfect match provides a strong evidence of the validity of the gene expression analysis. This sequence has been submitted to GenBank and assigned Accession number DQ388452.

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1 agg aga gcc tca act ctt gag gtc aac cca caa gca cgc caa agg ctc cag gag ctc ttt
R R A S T L E V N P Q A R Q R L Q E L F
61 gtg att ttc tgc ctg atc tta att tgc ctc ttg ctg atc tgt atc att gtg atg ctc ctc
V N F C L I L I C L L L I C I I V M L L
121 tga agt tcc agc act tct ctg cac tgt tct cta gga aag ccg ccc aaa ggg aag agc gac
* S S S T S L H C S L G K P P K G K S D
181 ctc cac ttc caa ccc caa tgc aag gat ccc ctt ggg aga ggg gct agc act tgg act aag
L H F Q P Q C K D P L G R G A S T W T K
241 gct gtg ctg tgt gcc agc cat cgc gct gct ctt act gcc 278
A V L C A S H R A A L T A

```

Figure 6. Translated sequence of turkey phospholamban cDNA sequence using the Open Reading Frame Finder (Büssow et al., 2002). An extra base of thymine (T) is inserted at the 121st of base (the bold letter). This translated sequence has a stop codon (M) with the green highlight and a conserved region with the red highlight.

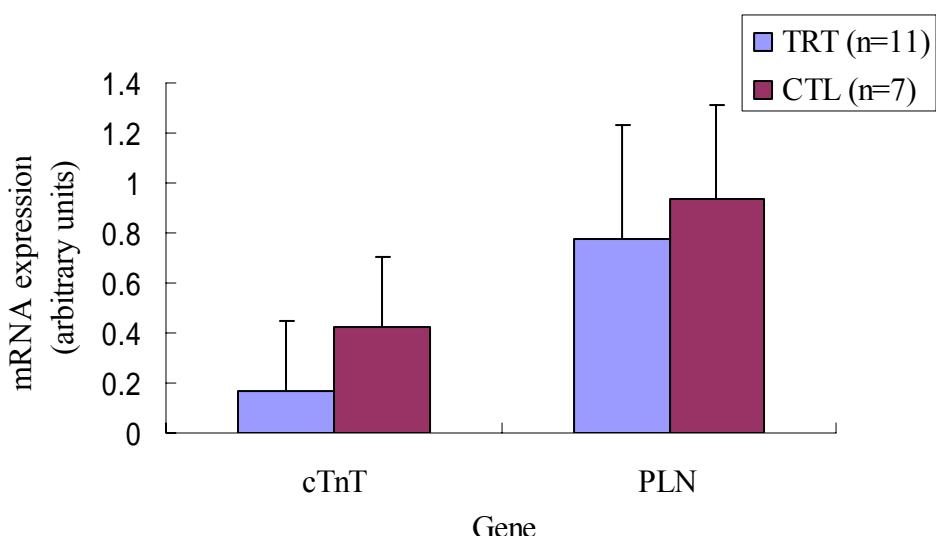


Figure 7. Cardiac troponin T (cTnT) and phospholamban (PLN) mRNA expression in Fz-induced (TRT) and normal (CTL) relative to β -actin, calibrated to the CTL group in heart. There were not significant difference between TRT and CTL birds in cTnT and PLN, $p = 0.5319$ and 0.8272 , respectively. Arbitrary unit represents log-transformed fold.

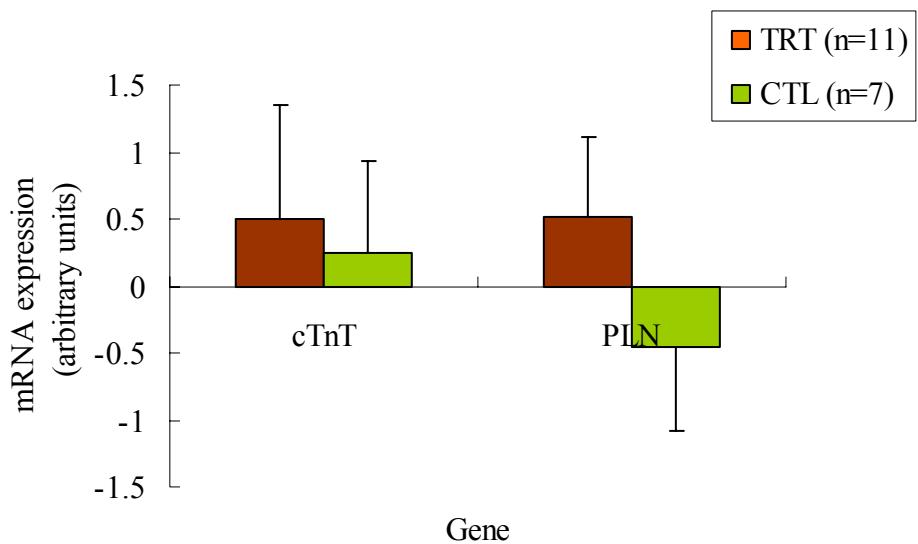


Figure 8. Cardiac troponin T (cTnT) and phospholamban (PLN) mRNA expression in Fz-induced (TRT) and normal (CTL) relative to β -actin, calibrated to the CTL group in liver. There were not significant difference between TRT and CTL birds in cTnT and PLN, $p = 0.8124$ and 0.2804 , respectively. Arbitrary unit represents log-transformed fold.

CHAPTER 4

SNP analysis of candidate genes for toxin-induced dilated cardiomyopathy in the turkey (*Meleagris gallopavo*)

4.1 ABSTRACT

Cardiac Troponin T (cTnT) and phospholamban (PLN) genes have been implicated in the incidence of idiopathic dilated cardiomyopathy (DCM) in vertebrates. Here, we scanned these genes for single nucleotide polymorphisms (SNPs) and developed haplotypes that could be useful in evaluating their roles in the incidence of toxin-induced dilated cardiomyopathy in turkeys. The turkey cTnT and chicken PLN sequences in GenBank were used to design primers used in the genome analysis. A DNA panel of 55 samples from 5 turkey strains was used to scan for SNPs. Additionally, one of the SNPs in the cTnT was used to develop a polymerase chain reaction-restriction fragment length polymorphism method to genotype the University of Minnesota DNA resource panel for mapping. A total of 1,069 and 666 bp of cTnT and PLN genomic sequences, respectively were scanned for SNPs. Within these sequences, 12 SNPs were identified in the cTnT and PLN genomic sequences. Eight haplotypes were observed from 7 cTnT genomic SNPs. Linkage analysis based on one of the SNPs showed that cTnT is currently unlinked. The frequency distribution in the six turkey strains was significant for only one of the haplotypes.

Keywords: Turkey, Toxin-induced cardiomyopathy, Cardiac troponin T, Phospholamban, Single nucleotide polymorphism

4.2 Introduction

With the completion of sequencing of genomes of many organisms and the development of high-throughput SNP genotyping systems, identifying the role of genomic variation in disease risk has become relatively easier (Niu et al., 2002; Wang et al., 2005). An important requirement in disease gene analysis is to identify genetic markers such as SNPs (Wang et al., 2005). The advantage of SNPs include abundance and stability in an organism, and the ease of developing highly automated analysis systems to genotype individuals for informative SNPs. These advantages make SNPs markers of choice for mapping and identifying disease genes, population genetics, and molecular evolution (Gibbs et al., 2003; Trikka et al., 2006).

In addition to SNPs, haplotypes, which combine several SNPs, are useful to map genes for complex diseases. Haplotypes provide a statistically useful and more reliable tool to conduct association studies using linkage disequilibrium analysis. Olson and Keating (1996), for example, observed haplotypes that were associated with inherited DCM. They used linkage and haplotype analyses to map the locus of one of five SNPs to a 30 cM region of chromosome 3. Other studies that have identified SNPs and haplotypes associated with DCM include those described by Towbin et al. (1993) and Muntoni et al., (1993) in the dystrophia genes.

The primary objective of this study was to scan PLN and cTnT for SNPs and if possible establish haplotypes.

4.3 Material and Methods

4.3a Animals, Blood, and DNA

A total of 55 birds from five turkey varieties including Blue Slate (n= 8), Bourbon Red (n=11), Narragansett (n=8), Royal Palm (n=10), and Spanish Black (n=9) and a commercial turkey (n=9) were used to scan for SNPs. These turkeys were fed *ad libitum* and maintained as described in chapter 3. Blood was collected from each bird by brachial venipuncture in tubes containing 0.5M EDTA as anticoagulant. Each sample was divided into 50 µl aliquots and stored at -20°C if not used immediately. From each aliquot, genomic DNA was isolated using the salting-out procedure (Sambrook and Ruessell, 2001). Briefly, the following were added: proteinase K, TNE lysis buffer, and 10% SDS followed by incubation overnight at 37 °C. Genomic DNA was precipitated after 6M NaCl using 3M NaAc and cold 100% ethanol. The precipitated DNA was dissolved in H₂O and stored at 4°C.

The DNA concentrations were determined by measuring absorbance at 260 nm and DNA quality was verified by electrophoresis on 2% agarose gels made with 0.5X TBE buffer (Sambrook and Ruessell, 2001).

4.3b Molecular analysis

Primers used in the PCR were designed by Primer 3 program computer program (Rozen and Skaletsky, 1997). The turkey cTnT gene, Accession number

AF374417, was design primers to produce overlapping products that were scaned for SNPs. To obtain turkey PLN amplicons to scan for SNPs, the primers used were developed from the chicken PLN sequence determined to be conserved in a Clustal-W-based multiple sequence comparison. Each primer was optimized for annealing temperature and reaction conditions using the FailSafe PCR PreMix Selection kit (Epicentre Inc., Madison, WI). Each amplicon was gel purified and sequenced using the BigDye terminator sequencing protocol (ABI, Foster City, CA). The sequences were analyzed by Phrap (for assembly of the sequences), Polyphred (for scanning the traces and Consed (for viewing the analysis) as described by Gordon et al. (2006). Because both cTnT and PLN have not previously been mapped on the turkey chromosome, we used the Minnesota mapping reference panel (Knutson et al., 2004) to conduct linkage analysis.

For PCR-restriction fragment length polymorphism (PCR-RFLP), the PCR amplification was run at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 62°C for 45 s, and 72°C for 1min by using PCR master mix kit (Promega). 5 ul of the amplicons was run on the 2% agaros gel containing ethidium bromide (EB). Twelve ul of the amplicons was used for RFLP analysis. The rationally existing restriction enzyme *ApaL* 1 site in the cTnT was identified using the software JustBio (<http://www.justbio.com/>). The recognition sequence was G^TGCAC which is that for

SNP. Following PCR, each amplicon was digested with *ApaL* I restriction enzyme according to manufacturers (New England Biolabs, Inc., USA) recommended protocol. The digested products were run on the 2% agarose and stained with ethidium bromide.

4.3c Haplotype frequency and linkage analyses

The frequency distribution of haplotypes was determined by counting and tested for significance using the Chi-square test. To test for linkage, the genotypes of the samples in the Minnesota panel were analyzed as described (Harry et al., 2003). Briefly, parental samples were scanned for informativeness for the PCR-RFLP marker, followed by genotyping of the F2. Pairwise recombination estimates and testing significance or LOD scores were according to the parameters of CriMap (Green et al., 1990).

4.4 Results and Discussion

The sizes of the amplicons produced by primers from cTnT and PLN were as expected (Figures 4.1 and 4.2). The amplicons produced by the two cTnT-based primer-pairs were 989 and 865 bp for primer-pair cTnT199 and cTnT267, respectively. The PCR-products from the chicken PLN gene-based amplification of turkey genomic DNA were 833 and 938 bp, respectively. The sequences of the amplicons have been submitted to GenBank and assigned accession numbers DQ435309 and DQ435310.

BLAST-based analysis of sequences of the cTnT amplicons showed sequence similarity of greater than 97% (Figure 4.3). This high sequence similarity provided additional support for the fidelity of PCR and the subsequent SNP analysis. It also provided further credibility of the GenBank-based cTnT genomic DNA sequence. The sequence analysis showed that the region of cTnT scanned for spans four exons (exon7 through 10), and three introns. Comparative analyses of the cTnT sequence showed significant match with the chicken cTnT and a map location of chromosome 32 (Figure 4.4). The sequences of chicken PLN-based turkey amplicon also showed significant BLAST-based sequence similarity with reference GenBank sequences. Though the sequence of the amplicon from PLN primer pair PLN16a matched the reference cDNA sequence (86% sequence identity, Figure 4.5), the sequence of the amplicon of PLN primer pair PLN16b did not. Both sequences, however, showed

significant sequence similarity to chicken genomic DNA sequences (Figures 4.6 and 4.7). A total of 12 SNPs were detected in the Consed analysis (Figure 4.8). Five SNPs were in the PLN gene and seven in cTnT. Observed SNPs within cTnT gene formed 8 haplotypes (Table 4.3). The frequency distribution of one of the haplotypes, G-C-C-A-C-C-G, in the turkey populations from the Virginia Tech farm was significantly ($P<0.001$) different. This indicates that the frequency distribution of haplotypes among our turkey samples was not in Hardy-Weinberg Equilibrium. This result indicated that the frequency of haplotypes in our turkey population may have been affected by selection, mutation, or migration. It may be possible that this particular haplotype was predominant during natural or artificial selection (Falconer and Mackay, 2002).

A SNP, VTkcSNP4, was applied to genotype the turkey panels from University of Minnesota using PCR-RFLP (Figure 4.5). Eighty out of 95 birds in the panel were successfully genotyped. The efficiency of genotyping was 84%. Data analysis for linkage disequilibrium showed no significant linkage ($LOD < 3$) on the current turkey genetic map. The lack of linkage may be due to low density of the turkey map since the average distance between linked markers is 6 cM (Harry et al., 2003). Thus, because of a low resolution, the cTnT showed no significant linkage.

Though turkey cTnT cDNA and genomic DNA sequences have previously

been described, no extensive population analysis of variants has been done. The SNPs described here, however, are novel and represent genomic reagents that could be useful for future investigations involving cTnT. These SNPs are distinct from the non-synonymous nucleotide variant described by Biesiadecki et al. (2004) in one of the three subunits of the troponin complex, troponin I. An amino acid substitution, also known as R111C polymorphism that involves Arg and Cys residues lower troponin I binding affinity to troponin T, the molecule investigated in the present work. Since the variants identified here do not affect changes in amino acid residues, they are not expected to influence the troponin complex's role in regulating cardiac muscle contraction. Their value, however, could be in their possible association with causative SNPs. This potential value underlies the large-scale development of SNPs in many species. Currently, a SNP map for chicken is available for linkage studies and studying economic traits such as egg production and disease resistance (International Chicken Polymorphism Map Consortium, 2004).

In summary, a total of 12 SNPs were identified in cTnT and PLN genomic DNA; 8 haplotypes were observed from 7 SNPs within cTnT gene; haplotype 5, G-C-C-A-C-C-G, was the most frequent in the unaffected turkeys genotyped. The VTkcSNP4-based linkage analysis showed that cTnT is unlinked on the Minnesota turkey genetic map.

Table 7. Sequences of primers used in the polymerase chain reaction

Gene	ID	Accession No.	Primers	Amplicon length (bp)	Tm* (°C)
cTnT	cTnT199	AF374417	Forward 1: 5' GAAGATGAA ACAAAAGCACC 3' Reverse 1: 5' TGAGTGCTTGTGGAGGACAG 3'	989	62.4
	cTnT267		Forward 2: 5' AACAAAGAAGGCAGTGCTGGT 3' Reverse 2: 5' GGGAGGCACCAGGTTGGCAT 3'		
PLN [†]	PLN16a	NW_060336	Forward 1: 5' TGGGTTCAAGTTGACATTTG 3' Reverse 1: 5' ATGTAATGCAAGCACAGTGG 3'	833	60.5
	PLN16b		Forward 2: 5' TTGGTTTCTGCATTCA 3' Reverse 2: 5' GAAGGCATTCACCTTGTCT 3'		

*Tm represents the optimized annealing temperature at which a single amplicon of the expected size was obtained.

[†] The primers are derived from chicken PLN.

Table 8. Single nucleotide polymorphisms and sequence context in turkey cTnT and PLN sequences

Gene/Accession No.*	SNP ID	Sequence Context [†]	Position [#]
cTnT/AF374417	VTkcSNP1	CAAAC(G/A)CAGTG	411
	VTkcSNP2	TTCCT(G/C)TTCGG	527
	VTkcSNP3	CTGTT(A/C)GGGGT	530
	VTkcSNP4	CATCA(G/A)TGCAC	585
	VTkcSNP5	ATGGA(C/T)GCCTC	617
	VTkcSNP6	AAGCC(C/T)TAAAAA	891
	VTkcSNP7	AGCCC(G/T)AAAAG	892
	VTkcSNP8	TTATA(C/T)CTTGG	132
	VTkcSNP9	ATTAA(A/G)CAGTG	243
	VTkcSNP10	TTCAG(C/T)AGTAT	354
	VTkcSNP11	TAAAA(C/T)AATCA	364
	VTkcSNP12	CTTAA(C/T)TCTCT	569

*Cardiac troponin T (cTnT) and phospholamban (PLN) genes correspond to the accession number in GenBank.

[†]Within each sequence context, alleles at the single nucleotide polymorphism (SNP) locus are presented in parentheses.

[#]Position of the variant nucleotide within the consensus sequences deposited in GenBank.

Table 9. Turkey cTnT haplotypes and their frequency distribution in random bred heritage turkeys

ID	Haplotypes*	Frequency [†]
Hap1	-A-G-A-G-T-C-G-	0.0368
Hap2	-A-G-A-G-C-T-G-	0.0368
Hap3	-A-G-A-G-C-C-G-	0.0547
Hap4	-A-G-A-G-C-C-T-	0.1637
Hap5	-G-C-C-A-C-C-G-	0.5452
Recombinant haplotypes		
Hap6	-A/G-G/C-A/C-G/A-C-C-G/T-	0.1273
Hap7	-A-G-A-G-C-C/T-G/T-	0.0182
Hap8	-A/G-G/C-A/C-G/A-C-C/T-G-	0.0182

*Haplotypes are based on the SNPs in Table 4.2 within cardiac troponin T (cTnT) gene.

[†]Haplotype frequency was determined in a total of 55 unrelated turkeys.

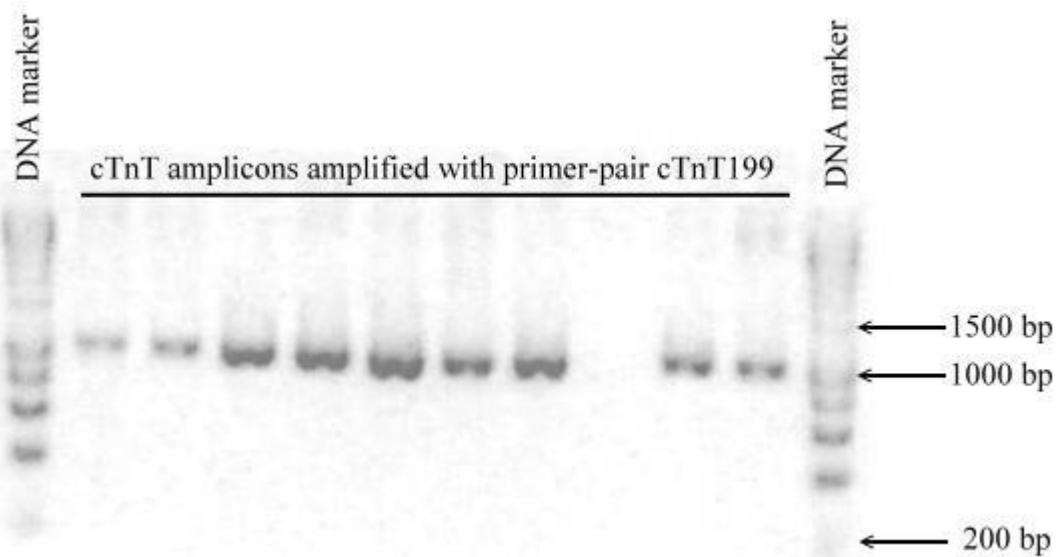
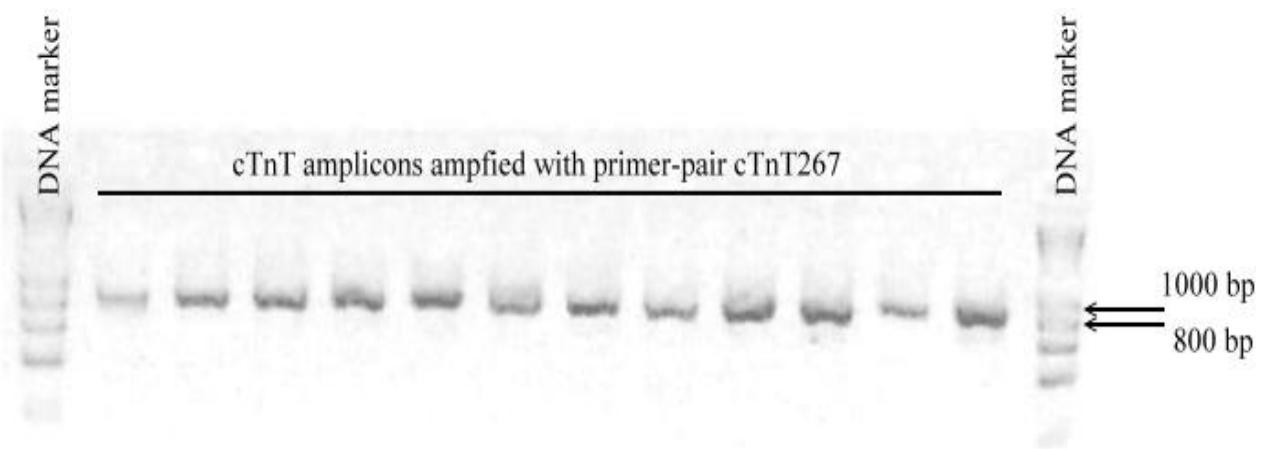
A**B**

Figure 9. SYBR Green-stained agarose gel patterns of turkey amplicons using primers-specific (Primer ID cTnT199 and cTnT267) for turkey cardiac troponin T (cTnT) cDNA sequence. Based on the predicted primer binding sites in the turkey, the expected PCR product sizes were 989 and 865 bp, respectively, in A and B patterns.

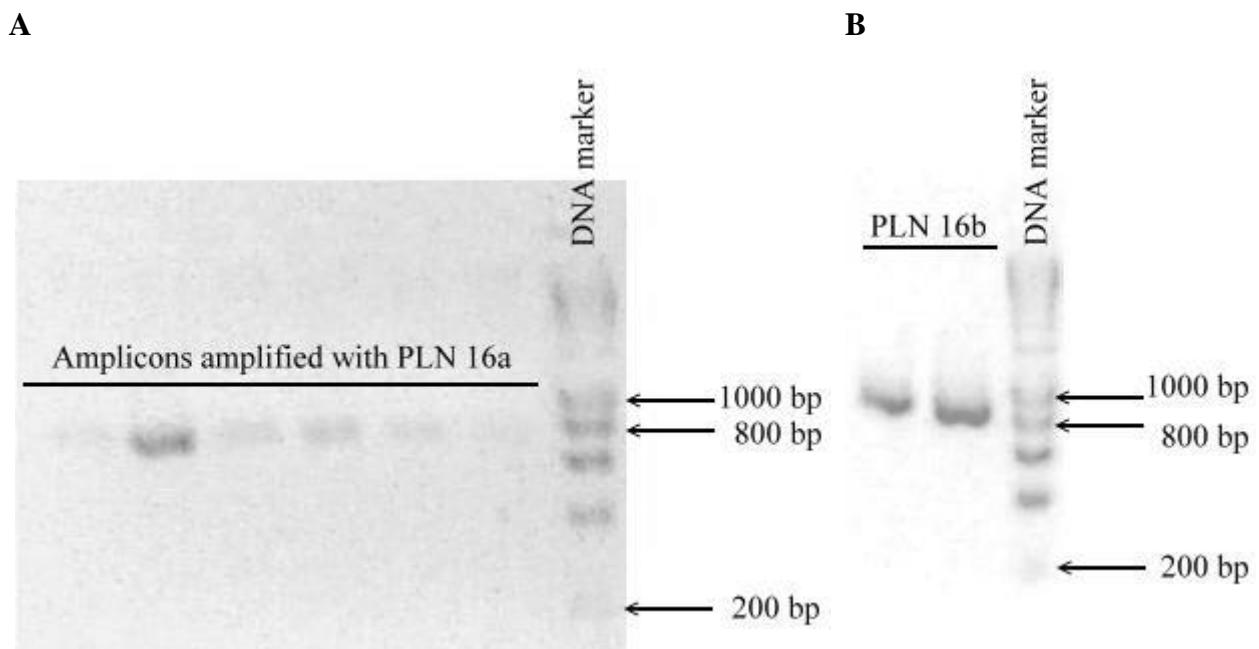


Figure 10. SYBR Green-stained agarose gel patterns of turkey amplicons using primers-specific for chicken phospholamban (PLN) DNA sequence. Based on the predicted primer binding sites in the chicken, the expected PCR product sizes were 833 and 938 bp, respectively, in A and B patterns.

Query Sbjct	1 55	TTTTGGCCATTCA-TCTCCCACAATGCTGCCCTGCCACGGGGTTTCAATGTTGA TTTTGGCCATTCAATCTCCCACAATGCTGCCCTGCCACGGGGTTTCAATGTTGA	59 114
Query Sbjct	60 115	ATTTTGAATTCACTGCTGCTGAAAGGAGACAATCTGCAGTAATTGGGCAGAGCTGCC ATTTTGAATTCACTGCTGCTGAAAGGAGACAATCTGCAGTAATTGGGCAGAGCTGCC	119 174
Query Sbjct	120 175	TGGCCCCATCCCTAGCAGGGCAGGGCAGCAGGAGATCTGCAGGAGGGATGTATTCATGC TGGCCCCATCCCTAGCAGGGCAGGGCAGCAGGAGATCTGCAGGAGGGATGTATTCATGC	179 234
Query Sbjct	180 235	TGCTTAGGTAAGAAATTCACCCGGAAATGAAACAAGAAGGCAGTGCTGGTGCCTCCC TGCTTAGGTAAGAAATTCACCCGGAAATGAAACAAGAAGGCAGTGCTGGTGCCTCCC	239 294
Query Sbjct	240 295	AGCCTGAGCTCCTGCAAAGCTCGTCTTGAGAGGACCCGGGGTCTCCCCTGCCTG AGCCTGAGCTCCTGCAAAGCTCGTCTTGAGAGGACCCGGGGTCTCCCCTGCCTG	299 354
Query Sbjct	300 355	GGGGTGAGGGGCCTGCCCTCCCATGTCCTCCTGGGCTTGTGGTGGGGCAAACGCAG GGGGTGAGGGGCCTGCCCTCCCATGTCCTCCTGGGCTTGTGGTGGGGCAAACACAG	359 414
Query Sbjct	360 415	TGCTGGGCAGCCTCACGGCCCTCCAGGGCCAACACATCCATGTTCCACAGGTGGTGA TGCTGGGCAGCCTCACGGCCCTCCAGGGCCAACACATCCATGTTCCACAGGTGGTGA	419 474
Query Sbjct	420 475	GGGGGACCGGGAGCAGGAGCCCAGGGAAAGGTGAGGCATCCATCCCTGTTCTTCGGGG GGGGGACCGGGAGCAGGAGCCCAGGGAAAGGTGAGGCATCCATCCCTGTTCTTAGGGG	479 534
Query Sbjct	480 535	TAGGAGGCACTGTCCTTCACAATACATAACCTGAATGCCCTGGAGCATCAGTCACGTCC TAGGAGGCACTGTCCTTCACAATACATAACCTGAATGCCCTGGAGCATCAGTCACGTCC	539 594
Query Sbjct	540 595	CAGATGTTGGCACACAGATGGACGCCACTGCATTGCTTGGTGGAGGATGATGTGCCT CAGATGTTGGCACACAGATGGACGCCACTGCATTGCTTGGTGGAGGATGATGTGCCT	599 654
Query Sbjct	600 655	GCACTGCTGAGAGGAAGGGGTGGTGGATTGGTGGGCTTGGGCTAAAGTATGCCACT GCACTGCTGAGAGGAAGGGGTGGTGGATTGGTGGGCTTGGGCTAAAGTATGCCACT	659 714
Query Sbjct	660 715	AACAATGCCCTTCCTCTGTCCTGGGATCCACAGGTGAATCAAAGCCAAACCCAAG AACAATGCCCTTCCTCTGTCCTGGGATCCACAGGTGAATCAAAGCCAAACCCAAG	719 774
Query Sbjct	720 775	TAAGTGTGGTGCACTTCTTGGTCAAGGTTGAACTAGGAGCTTCTATGGCT TAAGTGTGGTGCACTTCTTGGTCAAGGTTGAACTAGGAGCTTCTATGGCT	779 834
Query Sbjct	780 835	CTCACTGCAGCTTAGTGCCACAGTGAGATCCATAAGGAACGTGGACAGCAAAGCCCTAA CTCACTGCAGCTTAGTGCCACAGTGAGATCCATAAGGAACGTGGACAGCAAAGCCCTAA	839 894
Query Sbjct	840 895	AAGCAAGAAAACAGGGCAGGGACTGCAAGATGGAGAGAAGGGAGAAAAACAGAGAGAGAA AAGCAAGAAAACAGGGCAGGGACTGCAAGATGGAGAGAAGGGAGAAAAACAGAGAGAGAA	899 954
Query Sbjct	900 955	ACATGGGACTCTCCCTGCTCCACAAGCACTCATTGCTGGGGCTGTGGGCTCAGGG ACATGGGACTCTCCCTGCTCCACAAGCACTCATTGCTGGGGCTGTGGGCTCAGGG	959 1014
Query Sbjct	960 1015	AGGAGGGCTGGGAGAGCAGTGCAATGGTGGAGCCCTGCCCATCCCAGCTCCCCACTG AGGAGGGCTGGGAGAGCAGTGCAATGGTGGAGCCCTGCCCATCCCAGCTCCCCACTG	1019 1074
Query Sbjct	1020 1075	CCAGCTCCCAACCTCCCGCTCACCTCCAGGCCCTCATGGCAACCTGGT CCAGCTCCCAACCTCCCGCTCACCTCCAGGCCCTCATGGCAACCTGGT	1069 1124

Figure 11. BLAST-2-alignment of the turkey sequence (Query) of the amplicon produced using primers-specific (Primer ID cTnT199 and cTnT267) for turkey cardiac troponin T (cTnT), Accession No. AF374417, with GenBank turkey cTnT DNA sequence (Sbjct). The almost perfect match (98%) especially within the limits of the efficiency of sequencing, provide strong evidence of the validity of the SNP analysis.

Query Sbjct	1 321481	TTTTGGCCATTTC-ATCTCCCACAATGCTGCCTCCTGCCACGGGTTTCATGTTGA TTTTGGCCATTCAATCTCCCTCAACGCTGCCTGCCACGGGTTCTCAGTGTGG	59 321422
Query Sbjct	60 321421	ATTTGAATTCACTGCTGAAAGGAGACAATCTGCAGTAAATTGGG-CAGAGCTGC GTTTGAGTTCAGCCT-CTGCTGAAAGGAGACAATCTGCAGTAAA-TGGGACAGAGCTGC	117 321364
Query Sbjct	118 321363	CCTGGCCCCATCC-CTAGCAGGGCAGGGCAGCAGGAGATCTGCAGGAGGGATGTATTCA CCTGGCCCCATCCTGTAGCAGGGTAGGGCAGCAGGAGAGCTGCAGGAGGGATGTATTCA	176 321304
Query Sbjct	177 321303	TGCTGCTTAGGTAAGAAATTCACCCGGGAAATGGAACAAGA-AGGCAGTGCTGGTGCCT TGCTGCTTAGCTAAGAAATGCACTCCAGGAGATGGAACAAGACA-GCAGTGCTGGTGCCT	235 321245
Query Sbjct	236 321244	TCCCAGCCTGAGCTCCTGCAAAG--C-TC--G--T-CT-TGGAGAGGACCCGGGTTCT TCCCAGCCTGAGCTCCTGCAAAGCTCATCTGGGCTCCTCCTGGAGAGG-CCTGGGTTCT	286 321186
Query Sbjct	287 321185	CCCCATCTGCCTGGGGGTGAGGGG-CCTGCCCTCCCCATGTCTTCCTGGGCTTGTGGT CCCCATCTGCCTGGGTTG-GGGGATCTGCCCTCCTGTGTCCATCCTGGGCTTGTGGT	345 321127
Query Sbjct	346 321126	GGGGGCAAACGCAGTGTGGGCAGCCT-CACGCCCTCCAGGGCCAACACATCCATG GGGGACAAACG-GTGTGGGAAG-CTGCACAGCCTCTCCAGGGCCAACACATCCATA	404 321070
Query Sbjct	405 321069	TTCCACAGGTGGTGAGGGGGACCGGGAGCAGGAGCCCAGGGAAAGGTGAGGCATCCATCCC TTCCACAGGTGGTGAGGGGGACCGGGAGCAGGAGCCTGGGAAGGTGAGGCATTATCCC	464 321010
Query Sbjct	465 321009	TGTTCCCTCTTCGGGTAGGAGGCACTGTCCCTCACAAATACATACCCTG-AAT GCCTGGA TGTTCCCTCTTGTTGGGTAGGAGGTGCTGCCCTTCACAGTACATACC-TGGAACGCCAGGA	523 320951
Query Sbjct	524 320950	-GCATCAGTGCACG AGCATCAGTGCACG	536 320937

Figure 12. BLAST-2 comparison of the sequence (Query) of the amplicon produced by primers-specific (Primer ID cTnT199) for cardiac troponin T cDNA sequence, Accession No. AF374417. The DNA sequence was 88% identifies to a region of chicken chromosome 32 (Sbjct), Accession No.NW_060678.

Query Sbjct	131 3070	ACCTTGGTTGATTCTTGTCTTATTATCTTAAATGATTAATGGCTTGAGGTACAT ACCTTGGTTATTCTTGTCTTATTGTCTTAAATGATTAATGGCTTGAGGTACAT	190 3011
Query Sbjct	191 3010	AAGAAAAAAGCAGGCATTAACTTTATAGGAAGC--TTTTTCTTATTTAACAGTG AAGAGAAAAGCAGGTATTGATTTATAGGGAGGCTGTTTCTTTAGCAATG	248 2951
Query Sbjct	249 2950	TTCTGCTTACTACAAGCCTGTAGATCCTAGAACCTCAAATGATCTCAGAATGTGTAC CTCTGCTTCACTA-GGGCTGTAGGTCTAGGATCCCCAAATGATCTCAGAATGTGTAC	308 2892
Query Sbjct	309 2891	TGATATTTACTCTTAGCACCTTTCTTCAGAAAGTTTCAGCAGTATAAAACAATC TGATATTTACTCTGGCACCTTCCT-AGAAAG-TTTCA GTGCTATAAAACTGTC	368 2834
Query Sbjct	369 2833	AGTTATTCAGTAGAAATT TATT AAAATAAGTC 402 AGCTCTCCAGTAGAAGTGTACTGAAATAAGTC 2800	

Figure 13. BLAST-2-alignment of the sequence (Query) of the amplicon produced by primers-specific (Primer ID PLN16a) for chicken phospholamban (PLN). The sbjct is the chicken PLN cDNA sequence of Accession No. NM_205410. The alignment shows 86% sequence similarity to the region of the chicken PLN corresponding to nucleotide position from 3,070 to 2,800 bp. The turkey sequence has been submitted to GenBank and assigned Acc. No. DQ435309.

Query Sbjct	1	ACATTTGTGATGTGGA--GCATGGGAGCAGTTTCATATCACAGAACACTGAAGTGC ACATTTGTGATATGGAGTGCA--GGA-AAGTTCTCATATCAGCAGAAC-CTGAAGTGC	58	1610762
Query Sbjct	59	AAATGAACCTAAAATACTGCAATTACCTGTTGATGTAAAATCACAGTAGAGCTTC AAATGAACCCTACAATACTTGCATTCTGTGTTGATGTAAAATAACAGTAGAGCTTC	118	1610822
Query Sbjct	119	CAGCTTATTACCTGGTTGATTCTGTTGCTTATTATCTTAAATGATTAATGGCT CGGCTTATTACCTGGTTATTCTGTTGCTTATTGTCTTAAATGATTAATGGCT	178	1610882
Query Sbjct	179	TTTGAGGTACATAAGAAAAAGCAGGCATTTAATTATAGGAAGCT-TTTTT-CTT TTTGAGGTACATAAGAGAAAAGCAGGTATTTGATTTTATAGGGAGGCTTTTCTT	236	1610942
Query Sbjct	237	TATTTAACAGTGTCTGCTT GACTACAAGCCTGTAGATCCTAGAACCTC-AAAATGATC TATTAGCAATGCTCTGCTTCACTACGGGCTGTAGGTCTATAA-TCCCAAATGATC	295	1611001
Query Sbjct	296	TCAGAATGTGACTGATATTTACTCTAGCACCTTCTTCAGAAAGTTTCAGCA TCAGAATGTGACTGATATTTACTCTAGCACCTTCTTCAGAAAGTTTCAGT-	355	1611060
Query Sbjct	356	G-TATAAAACAATCAGTTATTCAGTAGAAATTAT TAAAATAAGTCCAATTAAATCTA GCTATAAAACTGTCAGCTCTCCAGTAGAAGTGTACTGAAATAAGTCCAAGTAATCTA	414	1611120
Query Sbjct	415	AAATGCCTCTATGAGAAAAGAAAG-ATGAAACCTTCCCACACATAATACAGATTAAA AAATGCCTGTATGAGAAAAGCAAGTATGAAACCTGCTATCACATAATAGATTAAA	473	1611180
Query Sbjct	474	AATAGAAGAAAGCAACTTTCTAT- TATT 502 AATAGAAGAAAGTAACATTATTG TATT 1611210		

Figure 14. BLAST-2-alignment of the sequence (Query) of the amplicon produced by primers-specific (Primer ID PLN16a) for chicken phospholamban. The sbjct is the chicken chromosome 3 sequence of Accession No. NW_060336. The alignment shows 88% sequence similarity to a region of the chicken chromosome 3 corresponding to nucleotide position 1,610,707 to 1,611,180 bp. The turkey sequence has been submitted to GenBank and assigned Accession No. DQ435309.

Query Sbjct	20343799	8 TCTGCATTCATCATGGCATGTATCTAC GTGACAGAGGAAGCACTAATTTCATTAATGT	67
Query Sbjct	20343857	68 CAGTTATGAACTCTACTGTAATTGAACGTGTTCTTGAAAGTGCTATCGCTATGGCT	127
Query Sbjct	20343917	128 TACAAAAACCTCCGGCATGAGGTAAATGACACGATTAACATTATAACAATTTCAGCTTGG	187
Query Sbjct	20343977	TACAAAAACCTCCGGCATGGGTAAATGACACGATTAACATTATAACAATTTCAGCTTGG	20343976
Query Sbjct	20343977	188 TATTCTTCCTATGACATGAAATGT GA-GTGCACGTGTCATTGTCATTGNNNNNNCATT	246
Query Sbjct	20344036	TATTCTTCCCAGACATCAAATGC -ATGTGACGTGTCATTGTCATTGTTTTTCATT	20344035
Query Sbjct	20344036	247 ATGTATGGCCTTTTACGGAACTAGGTGATTTCAT AATGTGAAAACAATGTGGTTATC	306
Query Sbjct	20344096	ATGTACGGCCTTTTATGGAACTAGGTGATTTCACAACGT GAAAACAATGTGGTTATA	20344095
Query Sbjct	20344156	307 TGCAACAACTTAAGACAGCGAACATGCCAGTTGACTGAATGTTCTAC AATAAAAAA	366
Query Sbjct	20344156	TGCAACAACTTAAGACAA ATGCCAGTTGGCTGAATGTTCTGCACGAAAAAA	20344155
Query Sbjct	20344216	367 CTGGGATTTCTGTTTTGTTCTCATGACTTTGACTCAATTCACTATTAAATGT TATA	426
Query Sbjct	20344216	427 TGGTGTGATCAGTATTAAATGGGGT CTGTAAAGAAGACAGAACAGGCTCCTAG	486
Query Sbjct	20344269	TGGTGTGATCAGTATTAAATGGGGT GATAAGAAGGAGACAGA---CT--CT--TTAG	20344268
Query Sbjct	20344269	487 CAGGGTCTGTTGACAGAACAAAGGT GAA-TGCCTT	521
Query Sbjct	20344269	TGGGGTCTGTTGACTGAACAAGGGAAATGG CCTT	20344304

Figure 15. A BLAST-2-alignment of the sequence of the turkey amplicon (Query) produced by primers-specific (Primer ID PLN16b) for chicken phospholamban (PLN), Accession No. NW_060336. The turkey sequence has been submitted to GenBank and assigned an Accession No. of DQ435310. The alignment shows a match with a sequence (Sbjct) in the region of chicken chromosome 3, which contains PLN with percent similarity of 90%. The chicken PLN is found around this region.



Figure 16. A. Consed-aligned view showing turkeys segregating for a G-A SNP (Arrow) in the cardiac troponin (cTnT) gene.

B. The trace view shows that the variant nucleotide is flanked on either side by more than three high-quality nucleotides.

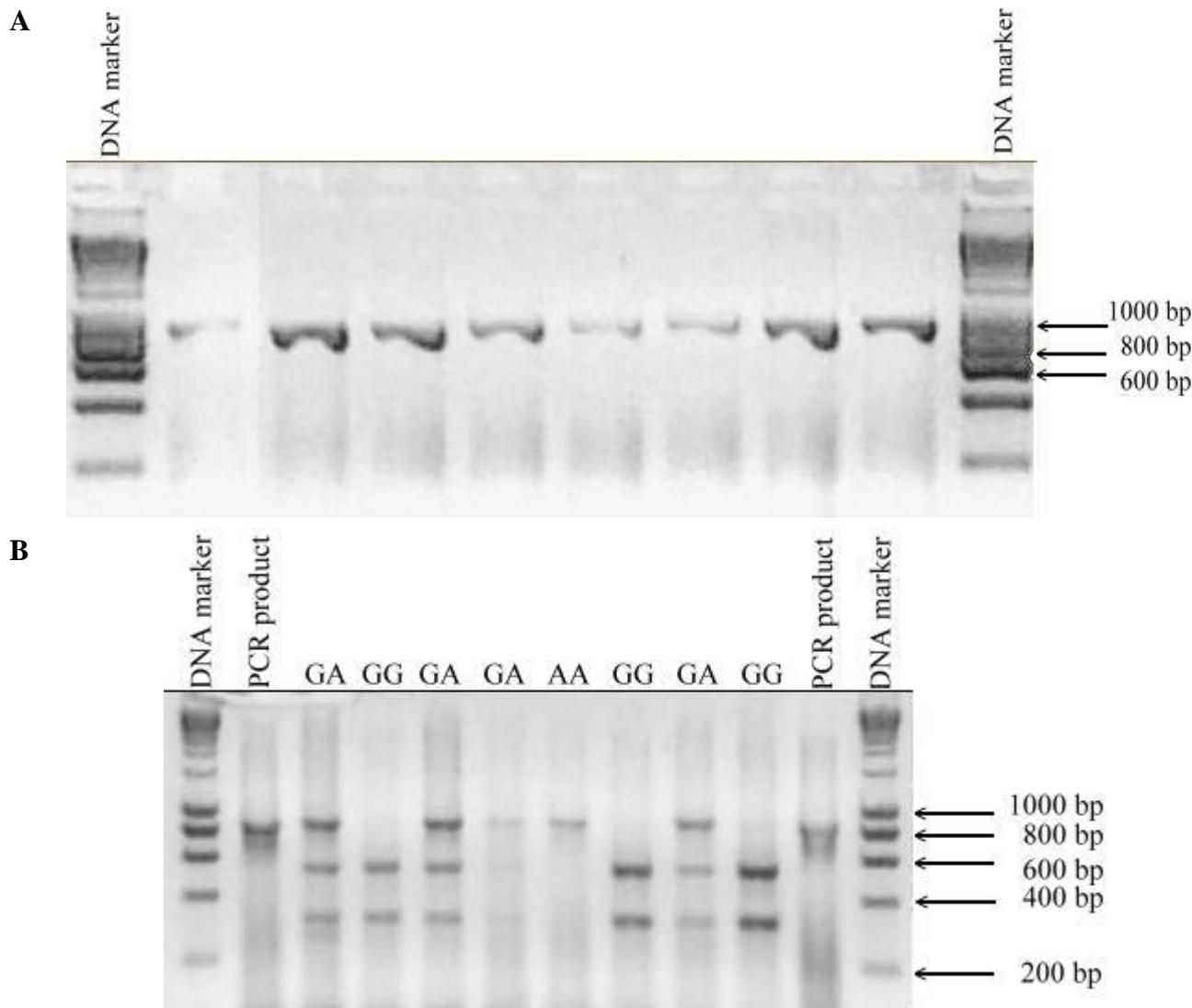


Figure 17. Agarose gel patterns after SYBR-Green staining of:

- A.** PCR amplicons produced using primers specific for turkey cardiac troponin T cDNA sequence.
- B.** *ApaL I* restriction digestion products of amplicons shown in **A**. GG, AA, and GA indicate digested products of amplicons from birds with homozygous, homozygous, and heterozygous genotypes, respectively, at the VTkcSNP4 locus.

CHAPTER 5

SUMMARY OF THESIS

This thesis research investigated the hypothesis that differential expression of candidate genes, including phospholamban (PLN) and cardiac troponin T (cTnT), affected the incidence and severity of induced DCM in turkeys. The preliminary studies determined that tissue but not blood is the most useful source for RNA for the expression analysis.

Specific conclusions are:

1. Changes in gene expression between DCM affected and normal turkey pourets were inconsistent and not significant.
2. A total 12 of SNPs were identified from cTnT and PLN; 8 haplotypes were observed from 7 SNPs within cTnT gene; one of haplotypes, G-C-C-A-C-C-G, was the most frequent in our turkey population and was not in Hardy-Weinberg Equilibrium.
3. Turkey troponin T is currently unlinked on the turkey genetic map.

Future work

Through the present work did not show that Furazolidone induced DCM is affected by cTnT and PLN gene expression, there remains a strong and unique opportunity in our laboratory to identify the molecular mechanisms that underlie toxin-induced. The novel resources developed, for example, will be essential for future

investigations to define the molecular factors that underlie toxin-induced DCM in the turkey. These resources, including the SNPs, the cDNA sequence, and haplotypes as well as the genotyping procedure will facilitate future association studies. Specific future investigations that could help our understanding of the molecular basis of these questions include:

1. Use microarray analysis to search for novel genes associated with toxin-induced DCM in the turkey.
2. Conduct association studies between the SNPs and haplotypes identified in PLN and cTnT and toxin-induced DCM.
3. Map cTnT and PLN so that a map-based approach can be used to associate the regions in which these genes are located with toxin-induced DCM in turkey.

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