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Molecular Diagnosis of Metabolic Fast Growth Related Diseases in Broiler

Adnan Ali Khalaf Al-Rubaye
University of Arkansas, Fayetteville

MOLECULAR DIAGNOSIS OF METABOLIC FAST GROWTH RELATED DISEASES IN BROILER

MOLECULAR DIAGNOSIS OF METABOLIC FAST GROWTH RELATED DISEASES IN BROILER

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By

Adnan Ali Khalaf Al-Rubaye
University of Baghdad
Bachelor of Veterinary Medicine, 2000
University of Baghdad
Master of Science in Medical Microbiology, 2003

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University of Arkansas

ABSTRACT

Pulmonary Hypertension Syndrome (PHS) and lameness are important metabolic diseases that affect rapidly growing broilers. The research reported in the first section of this dissertation focused on developing qPCR assays to identify differences in the expression levels of four candidate genes possibly associated with PHS: angiotensin II type 1 receptor (AGTR1): urotensin receptor 2D (UTS2D); serotonin receptor/transporter type 2Bn (HTR2B); and angiotensinogen cleaving enzyme (ACE). Expression levels of these candidate genes were examined in four different tissues. We established ribosomal protein S14 (RPS14) and RNA polymerase subunit 2B (RP2B) as suitable reference genes because they showed the most consistent deltaCt values as compared to TAT-Box binding protein (TBP) and β2-microglobulin (β2M). We found a wide range of expression variation of HTR2B and AGTR1 in blood compared to lung, liver, and kidney, indicating that blood expression levels of these candidate genes potentially will provide a minimally invasive assessment of susceptibility to PHS. Lameness attributable to Bacterial Chondronecrosis and Osteomyelitis (BCO) has been associated with a wide range of bacterial species. In the second section of this thesis 16S ribosomal RNA was used to identify bacteria causing BCO in broilers. *Staphylococcus* species were the main bacterial species isolated from BCO lesions and the blood. *Staphylococcus agnetis* was the principal species isolated from the majority of the bone and blood samples collected from lame broilers that had been hatched in two different hatcheries in two independent experiments. *Staphylococcus* species also were isolated from the blood of several apparently healthy birds.

This dissertation is approved for recommendation

to the Graduate Council.

Dissertation Director:

_____

Dr. Douglas Rhoads

Dissertation Committee:

_____

Dr. Robert F. Wideman, Jr.

_____

Dr. Yuchun Du

_____

Dr. Daniel Lessner

_____

Dr. Danny Davis

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DEDICATION

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I. Chapter one: Introduction

Commercial chicken production can be divided into two broad categories, broilers and layers. Broilers are meat producing chickens, and layers are egg producing chickens. Broilers and layers have been selected over many generations for different production characteristics, thereby leading to significant differences in their morphological and physiological characteristics. This dissertation focuses on broiler chickens. The main focus of broiler breeding companies has been and continues to be genetic selection for improved growth rates. In 1998, Anthony estimated that the time to produce 1 pound of broiler meat had decreased by 50% when compared to the 1950's, as shown in figure I.1 (Anthony, 1998). It has been estimated that 90% of the improvement in broiler growth rates can be attributed to genetic selection rather than to improved feed formulation (Havensteinet al., 1994a). These impressive improvements in broiler growth rates have led to the emergence of metabolic diseases that only become evident when broilers are grown at their maximum genetic potential. For example, exceptionally fast growth increases the workload of the heart causing cardio-pulmonary insufficiency in a subset of susceptible broilers. Cardio-pulmonary insufficiency in turn leads to the development of Pulmonary Hypertension Syndrome (PHS), also known as the ascites syndrome or ascites. Rapid growth rates also contribute to the development of lameness, presumably by placing excessive stress on the functionally immature leg bones and growth plates of susceptible broilers. A category of lameness known as Bacterial Chondronecrosis with Osteomyelitis (BCO) has emerged as the principal cause of lameness associated with fast growth and bacterial infection. Both PHS/ascites and BCO cause substantial financial losses for the broiler industry in poultry producing countries worldwide. The research reported in first section of this dissertation focused on developing qPCR assays to identify differences in the expression levels of four selected candidate genes associated with susceptibility to PHS. In the second section of this dissertation,

16S ribosomal RNA was used to identify bacteria causing BCO in broilers. These experiments represent an initial approach in which using molecular techniques were applied to better understand the pathogenesis of two important growth-related metabolic diseases in broiler chickens.

I.1 Broiler: Literature review

Broilers are raised mainly for their meat production due to their high ability to deposit more meat in less time as compared to layers. Due to intensive genetic selection during the past 50 years, broiler growth rates have increased dramatically. The total broiler production has increased from 366 million broilers in 1934 with an average live weight of 3.3 pounds per bird to 8.4 billion broilers with an average weight of 5.06 pounds in 2001. Increases in broiler meat production have been significantly higher than for other kinds of meat production. The total broiler meat production in 1945 was 1.11 billion pounds live weight, compared to 19.52 billion pounds of cattle and 18.84 billion pounds of hogs live weight. The production of broiler meat in 2001 was 42.45 billion pounds compared to 42.37 billion pounds of cattle and 25.94 billion pounds of hogs as shown in Fig I.2 (National Agricultural Statistics Service (NASS) & Agriculture, 2012). There are many factors that have led to the increase in broiler meat production in the United States, including improvements in poultry management, genetics, and vertical integration in order to keep pace with an increase in per capita consumption from 4.0 kg in 1950 (Tarver, 1986) to approximately 40 kg in 2000 (USDA, 2002). Broiler performance changed dramatically from 1923 to 2003, as shown in Table I.1 (Gordon, 1974; Havenstein et al., 2003).

I.2 Broiler industry in the United States

The broiler industry in the United States has been expanding and changing rapidly over the past fifty years. In 1934 11,450 hatcheries provided chicks to poultry producers, and the incubation capacity was 276 million eggs with an average of 24,244 eggs per hatchery. In 2001 there were 323 hatcheries with an incubation capacity of 862 million eggs and an average of 2.7 million eggs per hatchery as shown in Figure I.3 (National Agricultural Statistics Service (NASS) & Agriculture, 2012).

I.3 Broiler rapid growth advantage

The main characteristic of broilers is their ability to grow to large body weights in an extremely short period of time. A newly hatched broiler chick weighs 40gm and can reach up to 4000 gm in 8 weeks (R. F. Wideman 2013). The broiler industry has focused on increased broiler weight and decreased time required to reach to that weight. Recent scientific advancements in genetics and feed quality have allowed the broiler producers to accomplish their goals while maintaining excellent broiler meat quality and overall health. The main impact for the broiler rapid growth is the economic value that results from selling more pounds of broiler meat that cost less to produce. The total value for the broiler production has increased from $327 million in 1945 to $16.69 billion in 2001 (National Agricultural Statistics Service (NASS) & Agriculture, 2012). The two main factors that have contributed to the development of economically competitive broilers are their improved feed conversion rates, and their rapid growth rates, which result in decreasing the rearing time and the amount of feed required to achieve market weight (Cahaner, 2011). Genetic selection for fast growth has led to a tremendous increase in broiler body weights at younger ages.

I.4 Broiler rapid growth disadvantage

Over the past 50 years or more, broiler selection for rapid growth has led to a tremendous increase in broiler meat yield and overall improvements in feed conversion rates. Selection for rapid growth has led to huge economic gains, but at the same time it has led to the emergence of serious metabolic diseases. Heavy body weights accompanied by small skeletal frames in rapidly growing broiler have led to cardiovascular and musculoskeletal diseases (Julian, 1993; Lilburn, 1994; Riddell, 1992). The initial disadvantage attributed to rapid growth was the high deposition of fat in the carcass (Chambers, 1990; Soller & Eitan, 1984). Fat deposition affects meat quality and lowers the final sale price.

Another major problem caused by rapid growth is ascites syndrome or pulmonary hypertension syndrome (PHS). Susceptible broilers have an inadequate pulmonary vascular capacity that leads to the onset of pulmonary arterial hypertension followed by right ventricular hypertrophy, cardiac dilation and valvular insufficiency leading to right-sided congestive heart failure (Huchzermeyer & De Ruyck, 1986; Julian, 1993). In the 1980s the incidence of PHS began to increase rapidly in broiler chickens, mainly in association with high growth rates (Julian, 1998). The increase in growth rate was the a result of genetic selection and the high caloric diet which provided all the nutrients necessary for rapid growth and resulted in an increase in feed intake (Havenstein et al., 1994a). There are two types of PHS, primary and secondary, and broiler develop the primary form because there is no evidence of previous lung or heart disease (Julian, 1998). Fast growth in broilers leads also to decreases in oxygen hemoglobin saturation and higher incidences of ascites when compared to slow growing broilers (Julian & Mirsalimi, 1992).

Ascites in rapidly growing broilers at normal altitudes, can be prevented by growth rate restriction, primarily through feed restriction (Arce, Berger, & Coello, 1992). Ascites at high and moderate altitudes (above than 800 m) is a more severe disease because hypoxia in high altitude leads to polycythemia, but the incidence of PHS in high altitude can also be decreased by implementing growth rate restrictions (Julian, 1998).

Skeletal inadequacy leading to lameness is another problem related to rapid growth in broilers. Rapidly growing broilers demand more specific nutrients, and many skeletal deformities are rare or absent whenever broilers are grown relatively slowly (Havensteinet al., 1994a). Broiler lameness is a major welfare problem, and it seems likely that lame broilers experience pain when walking (Sorensen, 1989; Webster, 1994). Growth rate and live weight are important factors contributing to lameness in broilers (Kestin, Gordon, Su, & Sørensen, 2001). Skeletal disorders develop in male broilers at twice the incidence compared to females, probably because of the males' faster growth rate (Classen & Riddell, 1989; Riddell & Springer, 1985). A study of commercial broilers from 1971 concluded that long bone growth problems, especially those involving the growth plate, are the major cause of skeletal disorders causing lameness in broiler (Prasad, Hairr, & Dallas, 1972).

I.5 Pulmonary Hypertension Syndrome

Pulmonary Hypertension Syndrome (PHS) or ascites can be a major problem for the poultry industry especially in flocks with poor management (Franciosini, Tacconi, & Leonardi, 2012). The term PHS was first used by Huchzermeyer and de Ruyck (Huchzermeyer & De Ruyck, 1986). Ascites occurs mainly in the fast growing broiler flocks as compared to flocks in which growth is restricted (Dale & Villacres, 1988). PHS is known to occur in many other

animals including humans, pigs, and rats (Robbins, 2004). There is no full genetic map for the genes involved in the susceptibility or resistance to ascites. PHS affects the pulmonary vasculature of the bird as well as the heart muscle (Widemanet al., 2006). There are many factors that affect PHS in broiler, namely environment, genetics, and management. These factors affect oxygen demand and can forcing the heart to increase its output (Wideman et al., 2013). Our research entails identification of Quantitative Trait Loci (QTL) and subsequent identification of candidate genes that are responsible for causing PHS in broilers. This research focused on developing qPCR assays to identify differences in the expression levels of selected candidate genes and to examine expression levels in different tissues.

I.5.1 Ascites and Pulmonary Hypertension Syndrome, PHS

Ascites, pulmonary hypertension syndrome (PHS), or water belly is a metabolic disorder which characterized mainly by hypoxemia (low partial pressures of oxygen in arterial blood), increased central venous pressure, congestion and added pressure on the cardiopulmonary system (Luger, Shinder, Wolfenson, & Yahav, 2003), an accumulation of ascitic fluid in body cavities (Olkowski, Korver, Rathgeber, & Classen, 1999), right ventricular hypertrophy, and heart flaccidity (Riddell, 1991), and ultimately death of the affected bird (Luger, et al., 2003). Conclusions derived from many years of research on ascites is that ascites represents the final stage of a pathophysiological progression that is initiated by pulmonary hypertension. Ascites is also known as Idiopathic Pulmonary Arterial Hypertension (IPAH) and it is one of five kinds of pulmonary hypertension accepted by the American Lung Association. Our research group has developed a Quantitative Trait Loci (QTL) analysis to identify the genes that are linked to susceptibility to PHS in broilers.

I.5.2 Causes of PHS

In spite of the intense research on the etiology of ascites, the exact primary cause is still unclear (Crespo, 2003). The physiology of the PHS has been studied extensively by many researchers (Decuypereet al., 2000; Julian, 1993, 2000; Ladmakhiet al., 1997; Scheele, 1996; Wideman and Tackett, 2000). A wide array of factors interact to cause PHS in broilers, including genetic, physiological, environmental, and management factors as shown in figure I.4 (Baghbanzadeh & Decuypere, 2008). Ascites theoretically can develop in response to one or more of the following four physiological changes: decreased plasma oncotic pressure, obstructed lymph drainage, increased hepatic portal pressure which happens in response to right-sided congestive heart failure or primary liver damage (e.g., alcoholic cirrhosis), and fluid leakage caused by increased permeability of the peritoneal vascylature (Julian, 2005).

I.5.3 Ascites symptoms

The main symptoms of ascites or PHS include hydropericardium (fluid accumulation in the pericardium) and generalized edema, local extensive or focal adhesion between the visceral and parietal aspects of the pericardium, pericardium thickening, and fibrous deposits on the visceral part of the pericardium (Olkowski et al., 2003). Ascites is mainly characterized by the accumulation of ascitic fluid in the abdominal cavity (Balog, 2003), lung edema, fibrosis of the pericardium, and flaccid enlarged heart (Decuypere, et al., 2000). Ascitic broilers generally exhibit overt symptoms from pulmonary arterial hypertension including a noticeable hypoxemia that renders the comb and the wattles cyanotic (Julian & Mirsalimi, 1992; Peacock et al., 1989, 1990; Reeves et al., 1991; Wideman et al., 1998; Wideman and Kirby, 1995a, 1995b).

Hypoxemia, pale comb, variable liver changes, and elevated hematocrist have all been linked to PHS (Luger et al., 2003).

Right ventricular hypertrophy is one of the characteristic symptoms for PHS and it can be detected using electrocardiographic examination (Odom et al., 1992; Owen et al., 1995; Wideman et al., 1996, 1998; Wideman and Kirby, 1995a). Broilers that develop ascites can survive if diagnosed and rescued using diuretics like furosemide, which increases sodium and chloride excretion in the urine and eventually restores affected broilers' clinical health (Forman and Wideman, 2001; Wideman and French, 1999; Wideman and Kirby, 1995a).

I.5.4 PHS induction

In order to select against ascites susceptibility or to study ascites in general, researchers have developed experimental models to induce ascites (Balog, 2003). PHS can be induced using surgical and non-surgical methods. Surgical methods include obstructing one of the two pulmonary arteries. Surgical clamping of one pulmonary artery in male broiler chicks (age between 15-19 days) increases the pulmonary vascular resistance resulting in high incidences of ascites (Wideman and Kirby, 1995a). The permanent occlusion of one pulmonary artery leads to a primary increase in pulmonary vascular resistance (PVR) which results in the initiation of pathophysiological progression resulting in the development of ascites or PHS (Wideman et al., 1996; Wideman and Kirby, 1995a). Another surgical method to induce ascites is the unilateral occlusion of the primary bronchus. Unilateral bronchus occlusion is considered an effective experimental surgical way to trigger ascites at a lower incidence when compared with that obtained through the occlusion of one pulmonary artery. The increase of pulmonary arterial pressure (PAP) resulting from the occlusion of one pulmonary artery propels the overall cardiac

output at higher pressures and shear forces through the pulmonary vasculature (Wideman et al., 1997). Another effective technique to induce PHS is intravenous injection of microparticles. The microparticles then are carried to the lungs via the returning venous blood where they are trapped in the pulmonary vascular bed. Microparticle obstruction of the pulmonary vasculature initiates an acute in the PVR, pulmonary hypertension, systemic hypotension, and systemic hypoxemia (Wideman and Erf, 2002).

Non-surgical methods to induce ascites include cold stress models that involve raising the birds under a constant cold temperature (Julian, McMillan, & Quinton, 1989; Vanhooser, Beker, & Teeter, 1995). Induction of ascites through changing temperature can be done also through the gradual decrease in temperature (Buys, Scheele, Kwakernaak, & Decuypere, 1999), or through intermittent application of cold stress (Shlosberg et al., 1996). Another non-surgical way to induce ascites is through the use of a low ventilation model, which simulates a winterized broiler unit, but it is not as effective in inducing ascites as the cold stress model (Julian and Wilson, 1992; Shlosberget al., 1992). Another non-surgical technique to consistently induce ascites in broilers is the simulation of high-altitudes by using a hypobaric chamber (Anthony et al., 2001). The reduction of partial pressure of oxygen induced by operating under a partial vacuum creates conditions similar to those in natural high altitude. The use of this model has provided researchers with improved control of the temperature, ventilation, altitude, and humidity. Also, it allows researchers to repeatedly use consistent environmental parameters when the hypobaric chamber is used every time. As a result, using the hypobaric chamber is considered one of the leading, non-invasive, non-surgical models for inducing ascites in broilers (Pavlidis et al., 2007).

The hypobaric chamber technique was used as a selection tool to develop susceptible (SUS), resistant (RES), relaxed (REL) or control lines. Three separate groups of chicks that

experienced one generation of relaxed selection were transported to the University of Arkansas poultry research farm. These three groups of chicks represent the base population (Krishnamoorthy, 2012). At the 9th generation, the RES and REL lines showed a clear divergence in response to a simulated altitude of 9500 feet in the hypobaric chamber. The ascites incidence was 95% for the SUS line and 7% for the RES line (Pavlidis et al., 2007).

I.5.5 Genetic mapping of genes responsible for causing ascites

Many investigators have suggested that there is a genetic component behind the susceptibility to ascites (Anthony, et al., 2001; Closter et al., 2012; De Greefet al., 2001; Decuypere et al., 1994; Huchzermeyer et al., 1988; Jones, 1994; Lubritz et al., 1995; Pakdel et al., 2002, 2005; Pavlidis et al., 2007; Peacock et al., 1990; Shlosberg et al., 1996, 1998; Wideman and French, 1999, 2000). One of the most useful and modern techniques in the modern scientific revolution is to identify genes responsible for susceptibility or resistance for a disease. In ascites, there has been speculation regarding the number of genes that are responsible for the resistance or susceptibility to ascites (Wideman et al., 2013). Some researchers suggests that only a few major genes are involved in the susceptibility and resistance to PHS based on the fast progress achieved in the selection for PHS resistance and an estimated heritability of 0.4-0.5 (Wideman and French, 2000). An over-dominant gene model has been suggested based on 15 generations of blood oxygen saturation experimental data (Navarro, 2001). Several research groups have suggested that PHS is a complex polygenetic trait (De Greef et al., 2001;; Hamal et al., 2010; Rabie et al., 2005).

I.5.6 Genes responsible for PHS in broiler

To identify chromosomal regions linked to susceptibility to ascites, the RES and SUS lines were crossed to generate an F2 generation. The F2 generation was phenotyped for susceptibility or resistance to ascites in the hypobaric chamber (Pavlidis et al., 2007). A genome-wide panel of 3,072 SNPs was used to genotype DNA obtained from the F2 generation (Muir et al., 2008). The result from the genome-wide panel identified 4 chromosomes with 7 regions that are significantly associated with ascites phenotype (Wideman et al., 2013). There were 3 regions that showed association with ascites using multiple different lines (Smith, 2010). These regions are, based on the May 2006 *Gallus gallus* v2.1 genome assembly, chromosome 9:13.5-14.8 Mbp, chromosome 9:15.5-16.3 Mbp, and chromosome 27:2.0-2.3 Mbp (Wideman et al., 2013).

Four candidate genes in these three chromosomal regions were identified, based on physiological evidence, to the susceptibility to ascites in mammals and birds. These genes are: AGTR1, angiotensin II type 1 receptor; UTS2D, urotensin receptor 2D; 5HT2B, serotonin receptor/transporter type 2B;and ACE, angiotensinogen cleaving enzyme. Available research links each one of these genes to hypertension or hypoxic reaction in human or mouse (Chung et al., 2009; Djordjevic and Gorlach, 2007; MacLean, 2007; Simonneau et al., 2004; Watanabe et al., 2006).

I.5.7 Serotonin receptor, 5HT2B

Serotonin (5-hydroxytryptamine or 5HT) is a neurotransmitter with a strong pulmonary vasoconstrictive efficacy, and that also acts as a pulmonary artery smooth muscle mitogen (Eddahibi et al., 1999; Marcos et al., 2004). The etiology of PAH has been linked to serotonin metabolism (MacLean, 2007). An elevated level of serotonin has been recorded in patients who

developed IPAH as a consequence of a platelet storage disease (Herve et al., 1990). When binding to the 5HT2B receptor serotonin produces vasoconstriction, whereas the serotonin receptor and transporter (SERT) pathway mediates the serotonin mitogenic action (Eddahibi et al., 1999, 2002; MacLean et al., 2012).

I.5.8 Angiotensin II type 1 receptor, AGTR1

AGTR1 and angiotensin II have been shown to contribute to vasoconstriction, increased cardiac muscles contraction, hypertension, and cardiac hypertrophy in humans (Atlas, 2007). AGTR1 and angiotensin II play an important role in stimulating aldosterone secretion via the adrenal cortex, increase sodium reabsorption in the kidneys, and stimulation of the sympathetic nervous system (Atlas, 2007).

I.5.9 Angiotensin-converting enzyme, ACE

Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase, and its primary functions are to convert angiotensin I to angiotensin II, and to inactivate bradykinin (Iwai et al., 1994). ACE has been linked to primary pulmonary hypertension and right ventricular function (Abraham et al., 2003). ACE expression regulation plays a vital role in many cardiovascular diseases like hypertension (Okamuraet al., 1986).

I.5.10 Urotensin 2 domain containing, UTS2D

Human urotensin II is a potent vasoconstrictive peptide, and it plays an important role, together with its UT receptor, in causing hypertension in humans. UT II plays an important role in expediting the development of arteriosclerosis which leads to hypertension (Watanabe et al., 2006). High levels of UT II were found in patients with hypertension or diabetes type II (Cheung

et al., 2004). Urotensin II was first identified to have a vasoconstriction effect in mammals in rat thoracic aorta denuded of endothelium (Gibson, 1987). U-II is a strong human arterial and venous vasoconstrictor that has been tested and it has been linked to hypertension (Maguire et al., 2009). UT-II has been included among the strongest vasoconstrictors, with approximately 10-fold, 100-fold, and 300-fold greater strength than endothelin-1, serotonin, and norepinephrine, respectively, when evaluated in right arterial muscle from humans (Russell et al., 2009).

I.6 Lameness in broiler

I.6.1 Economic impact of lameness

Broiler leg problems including rickets, femoral head necrosis, tibial dyschondroplasia, and valgus-varus deformities, cost the United States broiler industry an estimated $120 million annually (Cook, 2000). According to a national survey of the broiler industry, broiler flocks experience an estimated 1.1% mortality, and an additional 2.1% of all broilers are downgraded or condemned during processing because of leg-related problems (Talaty et al., 2009). Severe lameness causes loss of body weight because lame birds are more hesitant to walk to feeders (McGeown et al., 1999). The overall integrity of the skeletal system is affected by many factors including: management; the environment; growth rate; genetics; nutrition; locomotive activity; age; toxins; and, infectious diseases (Rath et al., 2000).

Femoral head necrosis (FHN) is the major cause of lameness in commercial broiler flocks. FHN can be caused by an insufficient blood supply to the femoral head which leads to cell death in bone and bone marrow. As a consequence, the normal activities of osteoblasts and osteoclasts are disrupted, causing the femoral head to lose its structural integrity (Ditri et al., 2006). It was estimated that broiler lameness caused by FHN costs the UK broiler industry an

estimated £3.78 million a year (Pattison, 1992). In another study conducted in the UK, 61% of the birds exhibiting FHN also exhibited evidence of bacterial infection (Thorp et al., 1993).

It has been estimated that lameness caused by FHN in male broilers costs the Northern Ireland broiler industry an estimated £185,625 annually, while losses in female broilers due to lameness costs the Northern Ireland broiler industry an estimated £118,000 annually (McNamee et al., 1998).

I.6.2 Causes of lameness

Many factors contribute to lameness in broilers. However, the causes of lameness can be divided into 2 main categories: infectious causes, which are responsible for causing severe lameness, and skeletal abnormality causes, which lead to less severe lameness (Kestin et al., 1994; Lynch, Thorp, and Whitehead, 1992). Lameness also has been categorized into five groups based on the pathological factor that causes lameness: infectious disorders; nutritional disorders; metabolic conditions; conformational problems; and, toxins (Morris, 1993). The causes of lameness have further been categorized into three groups: developmental disorders; metabolic disorders; and, degenerative disorders (Riddell, 1992).

Femoral head necrosis (FHN) is a widely used term that describes necrotic lesions affecting the proximal femoral head (Bradshaw et al., 2002). This term was initially used in 1992 to describe infectious agents causing leg abnormalities (Reece, 1992). Bacterial chondronecrosis with osteomyelitis (BCO) currently is used to identify lameness caused by bacterial infections at multiple sites in a broiler's skeleton, including the proximal femoral head, the proximal tibial head, and the thoracic vertebrae (McNamee et al., 1998; McNamee and Smyth, 2000; Thorp and Waddington, 1997). Other terms related to BCO include osteomyelitis, proximal femoral

degeneration, long bone necrosis, bacterial chondronecrosis, and bacterial chondritis with osteomyelitis (Butterworth, 1999; McNamee and Smyth, 2000).

I.6.3 Incidence of lameness

FHN was reported as the most common cause of lameness in broilers in the UK (Pattison, 1992). Subsequently leg disorders were mainly attributed to BCO (Thorp and Waddington, 1997), and BCO now is considered to be the most common cause of leg problems in commercial broiler flocks worldwide(Bradshaw et al., 2002). In a study conducted on 28 flocks of male broilers and 19 flocks of females, the incidence of lameness was 0.52% in males and 0.52% in females (McNamee et al., 1998). A post-mortem study conducted on commercial broilers found that FHN/BCO was the most common cause of lameness at 38% followed by infection of the hock at 13.1%, twisted leg at 11.1%, and finally tibial dyschondroplasia (TD) at 7.2% (Pattison, 1992). Long bone deformities had been considered as the main cause of losses due to lameness in Canadian flocks, followed by osteomyelitis and arthritis at 10% (Riddell and Springer, 1985). Osteomyelitis/BCO was progressively recognized by the Farm Animal Welfare Council as the main cause of lameness in commercial broilers (Council and Britain, 1992).

I.6.4 Microbiology of lameness

Bacterial species reported to be involved in lameness caused by BCO include *Staphylococcus aureus*, *Eschericia coli*, and *Enterococcus cecorum*, frequently found in mixed cultures with other bacterial species like *Salmonella* spp.(Andreasen et al., 1993; Butterworth, 1999; Dinev, 2009; Joiner et al., 2005; Kense and Landman, 2011; McNamee et al., 1998; Nairn and Watson, 1972; Smeltzer and Gillaspy, 2000; Stalker et al., 2010; Tate et al., 1993; Thorp et al., 1993).

Staphylococcus aureus was reported as the most commonly isolated bacteria from broiler leg and joint infections (Skeeles, 1997). *Staphylococcus aureus* has been recovered frequently from bone infections in commercial broilers (Griffiths et al., 2008; McNamee et al., 1998; Nairn and Watson, 1972; Randall, 1996). There have been concurrent losses due to septicemia caused by *Staphylococcus* spp. in broilers affected by BCO (McNamee et al., 1998; Reece, 1992). Other bacterial species isolated from infected bones from broilers include: coagulase positive *Staphylococcus* spp. like *Staphylococcus hyicus*, coagulase negative *Staphylococcus* spp. like *Staphylococcuss imulans*, and *Staphylococcus xylosus, E. coli*, *Salmonella* spp., *Mycobacterium avium*, and *Enterococcus* spp.(McNamee et al., 1998; Reece, 1992; Thorp et al., 1993) .

Coagulase positive *Staphylococcus*, coagulase negative *Staphylococcus*, and *E. coli* or mixed cultures were recovered from 22%, 11.1%, and 13.3% respectively from broiler proximal femora diagnosed with BCO (Thorp, et al., 1993). *Staphylococcus* spp. was reported as the most common cause of arthritis, osteomyelitis, and tendonitis in broiler in Western Canada (Riddell, 1997). In a study conducted on 38 bones collected from broilers diagnosed with BCO, *Staphylococcus aureus* was diagnosed in 63.1% of the bones, non-haemolytic *E. coli* was diagnosed in 13.1%, *Staphylococcus xylosus* in 10.5%, *Staphylococcus hyicus* in 10.5%, *and Staphylococcus imulans* in 2.6% (McNamee, 1998).

As supported by a wide group of researchers, *Staphylococcus aureus* is considered as the common cause of lameness diagnosed as BCO in commercial broilers (McNamee and Smyth, 2000). *Staphylococcus aureus* is a Gram positive coccus that grows as characteristic grape shape aggregation. It expresses surface proteins that are responsible for its virulence, and its adherence to the host cells. There are many examples of *Staphylococcus aureus* virulence proteins like fibronectin binding protein, protein A, fibrinogen binding protein, collagen binding protein, and

bone sialo-protein (Foster and McDevitt, 1994; Holderbaum et al., 1985; Ryden, 1989; Switalski, 1993).

Staphylococcus spp. are considered normal flora of the poultry skin and mucous membranes, and are wide distributed within hatcheries, poultry houses, and processing plants (Skeeles, 1997). *S. aureus* was isolated from the skin and nares of birds (Harry, 1967a, 1967b), and from the plantar and dorsal surfaces of the feet of healthy chickens and wild birds (Cooper and Needham, 1976). As a consequence, wild birds are considered reservoirs to preserve and possibly transfer *S. aureus* to broiler chickens. Litter, feeders, drinkers, and poultry house air are other common sources for *S. aureus* (Sauter et al., 1981; Vaid et al., 1979).

I.6.5 Diagnosis of lameness

The primary clinical signs for lame broilers are: difficulties in standing, obvious limping gait with dipping one or both wing tips, and in more advanced cases, birds become completely immobilized. Lame birds with BCO die quickly (Dinev, 2009); therefore dead birds must be necropsied for any possible proximal femoral or tibial head lesions. BCO appears as localized lytic areas or yellow caseous exudate which makes the affected bone fragile (Skeeles, 1997). Lesions might appear as small pale areas next to the growth plate or as large yellow zones extended from the growth plate to the medullary space (McNamee et al., 1999). When examined histologically, BCO lesions usually appear as aggregation of basophilic bacteria in the physeal or epiphyseal blood vessels, surrounded by cartilaginous cartilages that are poorly stained and contain necrotic chondrocytes (McNamee and Smyth, 2000). Lame birds appear to be characteristically dehydrated and are smaller than other birds in the same flock (Emslie et al., 1983; McNamee et al., 1999).

I.6.6 Induction of lameness

Studying the pathogenesis, etiology, and treatment of BCO has been complicated because of the sporadic and low incidence of BCO in research flocks (Wideman and Prisby, 2013). Many researchers have tried inducing lameness in poultry through the intravenous injection of bacteria into the blood stream or through an aerosol administration. Intravenous injections of *S. aureus* and *E. coli* in turkeys have triggered the development of osteomyelitis that is similar to spontaneous osteomyelitis (Nairn, 1973). BCO was produced in broiler chickens using the aerosol route, and *S. aureus* was isolated from bone lesions in broilers that developed BCO (McNamee et al., 1999).

Recently a research model has been developed that induces BCO without exposing broiler to pathogenic organisms. This model is called the wire-flooring system (Wideman et al., 2012). This model has been very successful in triggering 20-60% lameness overwhelmingly attributable to BCO in broilers. BCO lesions produced by the wire-flooring system are obvious and include the presence of proximal femoral and tibial head necrosis (Wideman Jr, et al., 2012). The experimental procedure used for the present dissertation is based on inducing lameness using the wire flooring model, isolating and culturing the bacteria from BCO lesions and blood samples, followed by the molecular diagnosis of the bacteria using 16S ribosomal RNA.

Table I.1 Typical broiler performance in the USA (Gordon, 1974 and Havenstein et al.,2003).

Year	Week of age when sold	Live weight/kg	Feed efficiency kg feed/weight	Mortality %
1923	16.0	1.00	4.70	18.0
1933	14.0	1.23	4.40	14.0
1943	12.0	1.36	4.00	10.0
1957	12.0	1.43	3.84	4.7
1963	9.5	1.59	2.40	5.7
1973	8.5	1.77	2.00	2.7
2001	6.0	2.67	1.63	3.6

Figure I.1 Growth curve for the typical chicken in 1925 and meat type chicken in 1950, 1975 and 1990 with the arrows indicate the age at which slaughter was conducted (Anthony, 1995).

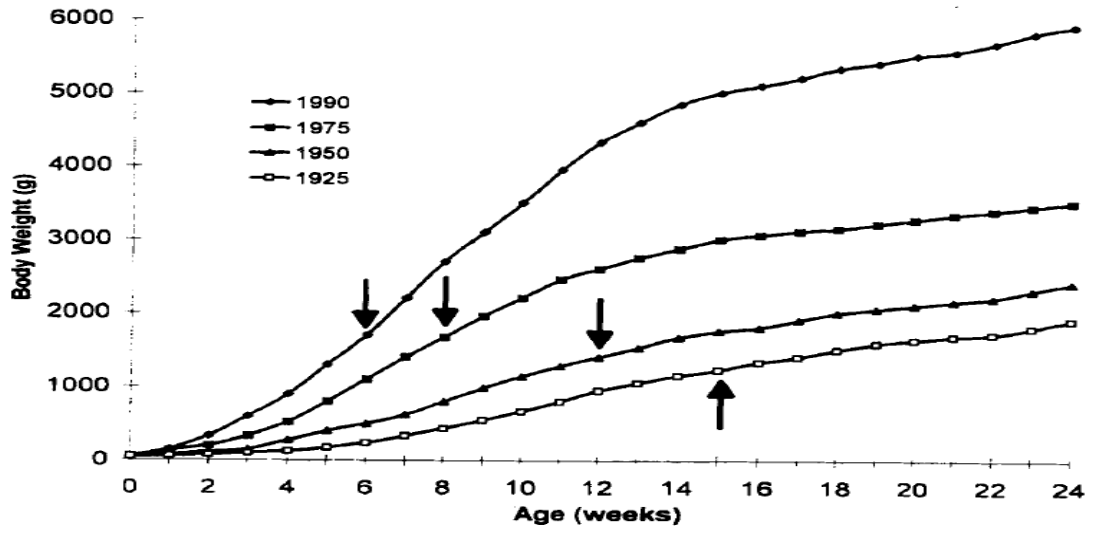


Fig I.2 Cattle, Hog, and Broiler Production in Pounds from 1945 to 2001 (National Agricultural Statistics Service (NASS) & Agriculture, 2012)

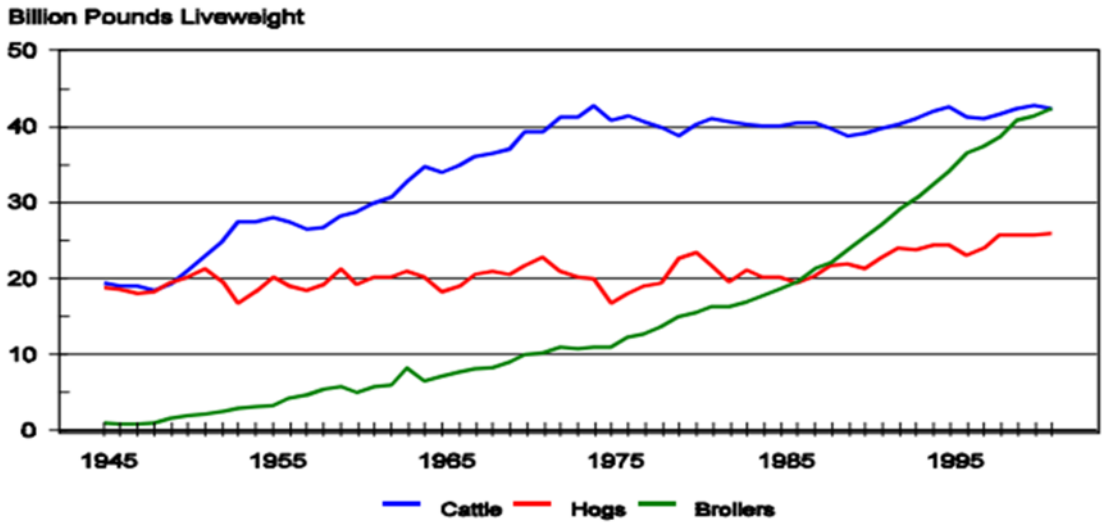


Fig I.3 U.S. Chicken Hatcheries 1934-2001(National Agricultural Statistics Service (NASS) & Agriculture, 2012)

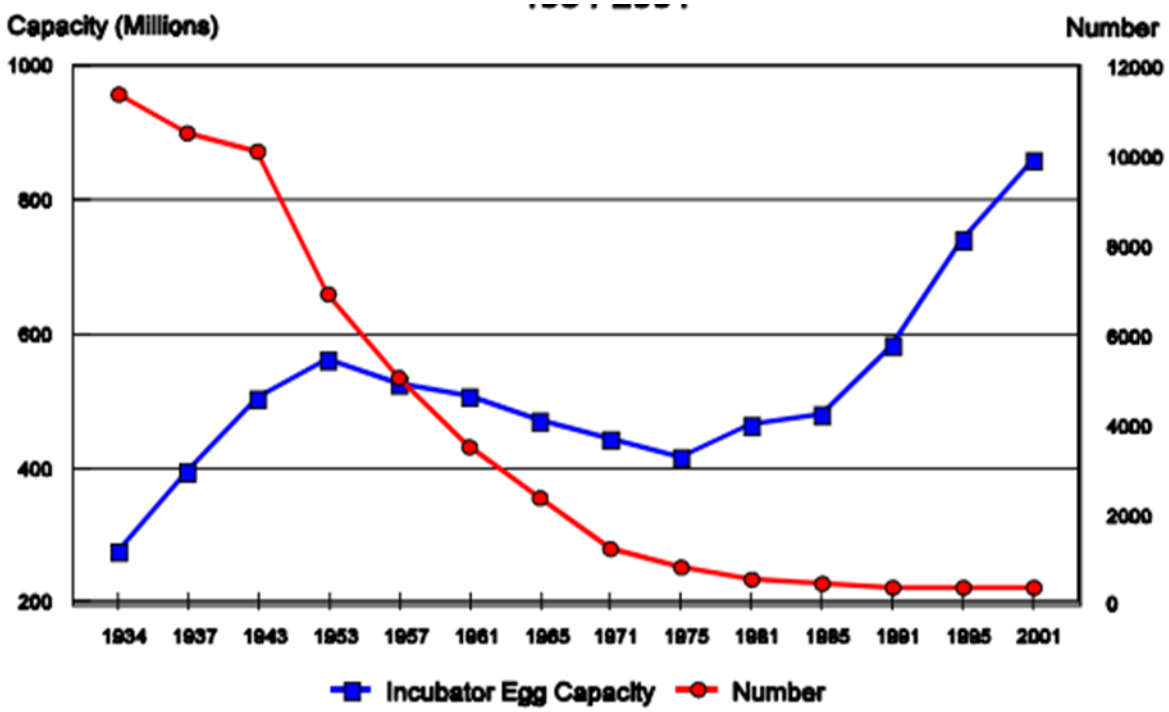
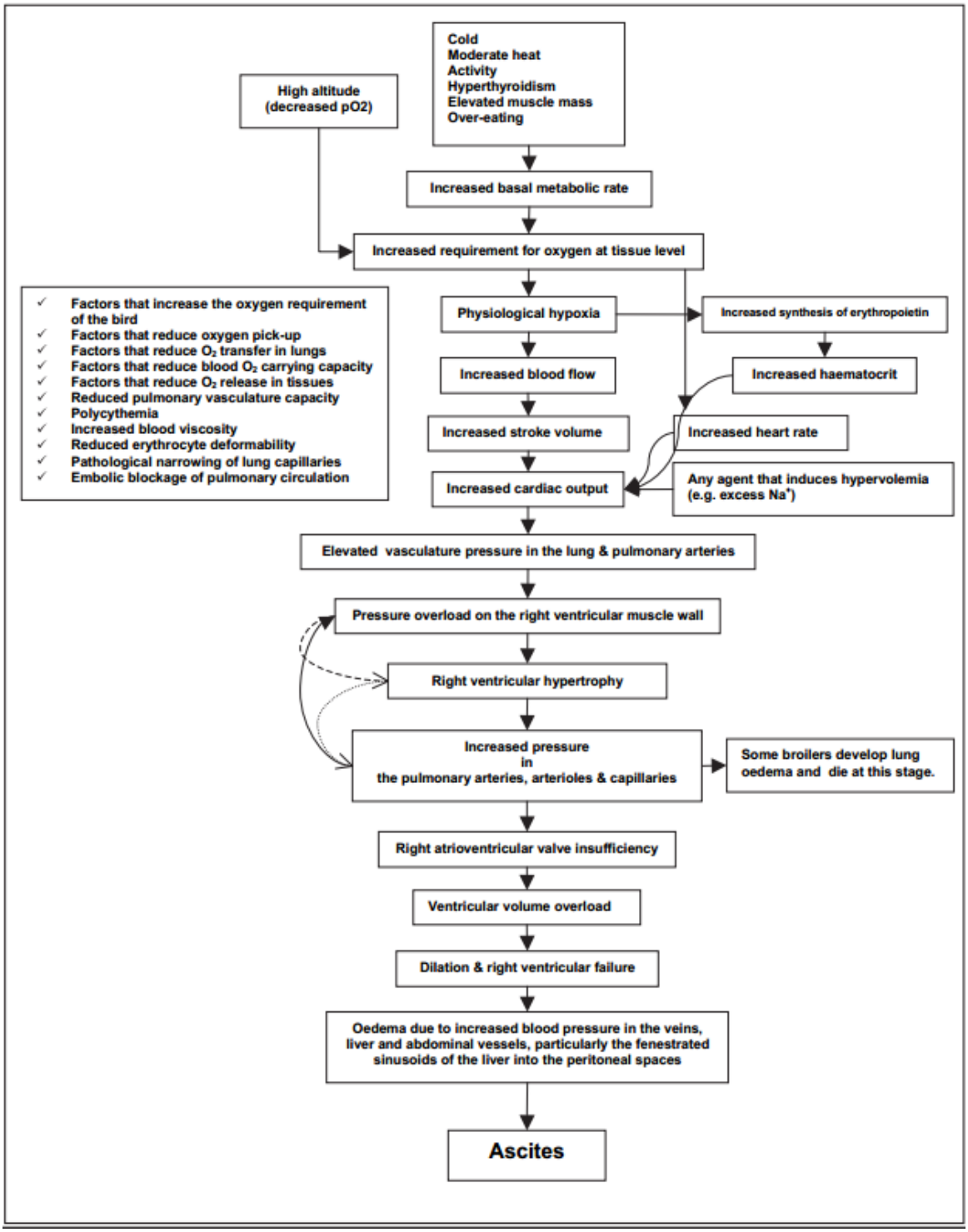


Fig I.4 Physiological and pathophysiological factors affecting ascites syndrome (Baghbanzadeh & Decuypere, 2008)



II. Chapter two: Genotypic and expression studies of genes associated with ascites in broilers

II.1 Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a disease of unknown cause characterized by elevated pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) attributed to vasoconstriction and vascular remodeling. Broiler chickens spontaneously develop IPAH characterized by increases in PVR attributable to pulmonary vasoconstriction and vascular remodeling. Broiler chicken lines have been developed that exhibit high heritability for IPAH susceptibility and resistance. Using the i.v. micro particle injection technique, a serotonin (5-hydroxy-tryptamine, 5HT)-mediated IPAH susceptibility has been demonstrated (Chapman and Wideman, 2006). In a genome-wide SNP-based comparison of an F2 cross between the lines, nine highly significant chromosomal regions associated with susceptibility and resistance to IPAH were demonstrated. Birds from the susceptible line that were highly susceptible vs. moderately resistant to IPAH were genotyped and six of the nine regions were validated for this population. Three of these chromosomal regions contain genes for Somatostatin-14 somatostatins interact with 5-HT to regulate vascular tone; HTR2B- (also called 5-HT2B) a high affinity serotonin receptor transporter; AGTR1- a type 1 angiotensin II receptor; ACE- angiontensinogen cleaving enzyme, and Urotensin 2 domain containing, UTS2D. Genetic polymorphisms in HTR2B, AGTR1, ACE, and UTS2D have been associated with spontaneous IPAH, anorexigen-induced IPAH, and PAH associated chronic obstructive pulmonary disease (COPD) (Chung et al., 2009; Eddahibi et al., 20012002, , 2003; Heils et al., 2002). Genetic analysis of IPAH in our avian model is directed toward identifying candidate genes that appear to correlate with human genes implicated in IPAH. Identification of the mutations and their

contributions to the etiology of the disease will be critical for development of the chicken as a medical model for human IPAH.

II.2 Materials and Methods

II.2.1 Experimental broilers

Broilers used in this experiment are resistant and susceptible birds, selected at the University of Arkansas, Poultry research farm, based on their abilities to develop ascites (Pavlidis et al., 2007). All birds received feed *ad libitum* and were raised under ideal environmental conditions of temperature, lighting, and ventilation until they started developing ascites (Wideman et al., 2003). Electrocardiogram evaluation and right ventricle to total ventricle (RV/TV) were used to evaluate birds' susceptibility or resistance to ascites. Lead II ECGs were utilized to diagnose the early onset of PHS. Birds are considered clinically healthy when their R and S waves have relatively small and equal amplitude. Birds were considered pre-ascitic when their R waves were small or non-existent with a large and deeply negative S wave amplitude. Birds with clinical PHS were rescued using light restriction, skip-a-day feeding, and by using furosemide to remove the accumulated ascitic fluids. Right heart hypertrophy was diagnosed using the RV/TV ratio in post-mortem examination. Blood and tissue samples were collected from resistant and susceptible birds, quick frozen on dry ice, and transferred to Dr. Rhoads' research laboratory in the Ferritor building, University of Arkansas.

II.2.2 RNA isolation

RNA was extracted using a guanidinium isothiocyanate method, adapted from Chomczynski and Sacchi (Chomczynski, 1987). A total amount of approximately 0.5 ml of blood was taken from each bird. Blood samples or approximately 1 gram of each tissue sample

were then homogenized with a Virtis hand held homogenizer in 5 ml of freshly prepared GUSCN. GUSCN was prepared by mixing 47.26 gm of GUSCN, 2.5 ml of 1M sodium citrate pH 7.0, 5 ml of 10% sarkosyl, and 54 ml of H2O. An amount of 0.5 ml of NaOac was then added to each sample. Thereafter, 5 ml of AE-Phenol was added followed by 0.5 ml of CHCL3: IAA. Samples were mixed very well and centrifuged using a high speed cold (4 ºC) J2-21 Beckman centrifuge at 10 K RPM for 20 minutes @ 4 ºC .The aqueous layer was transferred to a new tube, mixed with the same volume of isopropanol, and incubated at -20 ºC for 1 hour. Samples were then spun using @ 10k RPM for 20 minute @ 4 ºC. Following centrifugation, the aqueous layer was removed, and the pellet was dissolved in 500 µl of GUSCN, mixed well, and transferred into a 1.5 ml microfuge tubes. An amount of 500 µl of isopropanol was then added to each microfuge tube, and microfuge tubes were then allowed to stay at -20 ºC for 1 hour. After that, samples were spun down at 12k RPM for 15 minutes @ 4ºC. The aqueous layer was then removed, and the pellet was allowed to drain. Samples were then rinsed using 300 µl of 70% ethanol in cold centrifuge at 12k RPM for 5 minutes. The ethanol layer was removed, and the pellet dried using a vacuum centrifuge Savant Speed Vac SC110 for 5 minutes. The pellet was redissolved in 100 µl of Te, which was prepared by mixing 10mm Tris and 01 mm EDTA at pH 7.5.Final RNA concentrations were determined using NanoVue spectrophotometer.

II.2.3 cDNA synthesis

cDNA synthesis was conducted using a modification of the Gubler and Hoffman procedure. An RNA volume that is equal to 30µg was taken from each sample, ethanol precipitated in the presence of 0.3 M potassium acetate.

An amount of 10 µl of Te was then added to each sample. A cocktail of the following was prepared and was added to each sample: 20 µl 5× FS buffer (first strand buffer from Invitrogen), 1 µl of 20 mM dNTP, 2 µl of 30 µM first strand primer $T_{23}V$, 5 µl of 0.1 M DTT, and 57 µl of H_2O. Samples were then allowed to sit in 50 ºC water bath for 10 minutes. After that, we added 0.5 µl of RNasin from Promega, and 5 µl of SuperScript III (RNAase H^- MMLV reverse transcriptase from Invitrogen). Samples were then mixed well, incubated for 1 hour at 50 ^oC. We then added 100 µl of TE, 5 µl of 10% SDS, and5 µl of 250 mM EDTA into each sample. Samples were mixed well, and extracted with 200 µl of phenol-chloroform-isoamyl alcohol (50:48:2), then with chloroform-isoamyl alcohol (24:1). Samples were ethanol precipitated and redissolved in 200 µl of Te.

II.2.4 Quantitative Polymerase Chain reaction (qPCR)

The UCSC genome browser (http://www.genome.ucsc.edu/) was used to identify nucleotide sequences for HTR2B, AGTR1, ACE, and UTS2D. qPCR primer pairs were designed using Primer3 (http://frodo.wi.mit.edu/). A full list for primers used in this experiment is listed in Table II.1. Primers were also designed for four reference genes, TATA-Box binding protein (TBP), β2-microglobulin (β2M), ribosomal protein S14 (RPS14), and RNA polymerase subunit 2B (RP2B). Control genes were chosen based on the guidelines to reference gene selection for qPCR set by Aleksandar, 2003 (Radonić et al., 2004). qPCR was conducted in 96 well qPCR plates in a Bio-Rad CFX96 Real-Time System. Twenty microliter qPCR reactions were run using the following ingredients: 2 µl cDNA, in 1X SybrGreen Buffer (50 mMTris-Cl pH 8.3, 1mM MgCl2, 30ug/ml BSA, SybrGreen), 0.2 µM forward and reverse primers, 0.2 mM dNTPs, and 3 units Taq polymerase. The following qPCR conditions were used: 90°C for 30 seconds for the initial denaturation followed by 45 cycles of 90°C denaturation for 15 seconds,

30 seconds annealing at primer's optimum annealing temperature, and 72°C elongation for 30 seconds. Samples were run in triplicate and the average deltaCt value was recorded for each sample.

II.3 Results and discussion

Previous work in our group has confirmed the association of two regions on chromosome Gga9 and one region on chromosome Gga27 with PHS/ascites in the chicken (Fig II.1). A genome wide association study with a 3.4k SNP panel revealed that ascites phenotype and right ventricular/total ventricular (RVTV) phenotypes are statistically associated with two regions on chromosomes Gga9 and Gga27 (Fig II.2). RVTV is a measure of cardiac hypertrophy caused by the increased right ventricular work load required to develop an elevated pulmonary arterial pressure. As a consequence, RV thickening (muscle hypertrophy) will occur leading to an increase in the ratio between RV to TV (Julian et al., 1987; Julian and Mirsalimi, 1992; Peacock et al., 1990). The objective of the current project was to develop qPCR assays for measuring gene expression of candidate genes in each of these regions, and to evaluate variation of expression. Selection of candidate genes, described in the introduction, was done through the inspection of the two regions for genes associated with hypertension, vasoconstriction or hypoxia in humans.

For optimal determination of relative expression levels, it is crucial to use good reference or housekeeping genes for calculation of ΔCt values. The value ΔCt is computed as the difference in Cycle threshold (Ct) values between the reference and the experimental genes. We then compare ΔCt values between samples to ascertain where the experimental gene is over- or under-expressed. We measured Ct values for four different reference genes, and computed ΔCt between reference genes across multiple samples to discern which reference genes were the least

variable across multiple samples. Reference genes investigated were: TBP, β2M, RPS14, and RP2B. These reference genes were chosen based on the guidelines to reference gene selection for qPCR set by Aleksandar, 2003 (Radonić et al., 2004). Each reference gene was used to measure Ct values for different RNAs from blood samples from different chickens. Each sample was run in triplicate. The average Ct values then were used to calculate the ΔCt for each possible reference using the other candidate reference genes as the reference. Standard deviation of the ΔCt values for RPS14-Rp2B was the lowest (Table II.2). The rationale was that the best reference gene would have the most similar ΔCt values across multiple samples and thus the lowest standard deviation. RPS14 and RP2B were found to give the most consistent ΔCt values compared to TBP and β2M (Table II. 2). The standard deviation (STD) of the RPS14-RP2B ΔCt values was the lowest (2.2) of all comparisons, whereas the remaining ΔCt values exhibited standard deviations of 3.9 to 5.6. Therefore, across these various blood samples the RP2B and RPS14 reference genes appeared to show the least variation.

I then evaluated the numerous blood samples for ascites susceptible and resistant birds. Four candidate genes had been identified in the three chromosomal regions: AGTR1, HTR2B, UTS2D and ACE (Fig II. 2). AGTR1, HTR2B, UTS2D and ACE are known to be involved in vasoconstriction which can contribute to hypertension and ultimately lead to right ventricular hypertrophy and ascites (Chung et al., 2009; Djordjevic and Gorlach, 2007; MacLean, 2007; Simonneau et al., 2004; Watanabe et al., 2006; Heils et al., 2002). qPCR analysis for AGTR1 and HTR2B using RPS14 and RP2B as reference genes revealed a wide range of expression variation when compared across multiple whole blood samples (Table II. 3). The ΔCt values for ascites birds were all lower for both AGTR1 compared to the control (non-ascites) birds. The difference was observed whether I used RPS14 or RP2B as the references. These observations

suggest that there is 4 to 8 fold higher expression for these genes in ascites birds relative to the control, normal birds. This would indicate that the ascitic birds appear to be expressing AGTR1 at about 4-8 times the control level in blood (Figure II.3). Further work will be needed to confirm this important observation in additional birds, and to correlate genotypes for AGTR1 with ascites susceptibility or resistance. No consistent variation was seen for HTR2B (Table II.3 & Fig II.3).

I was unable to generate a reliable assay for ACE, but after the initial work on HTR2B & AGTR1, I was able to develop an assay for UTS2D. The HTR2B, AGTR1 and UTS2D qPCR were used on cDNAs from kidney, lung, and liver. Liver was considered a control tissue while the kidneys regulate blood pressure and osmolality, and the lungs are a primary organ responsible for the pathogenesis of PHS. Relative expression values as ΔCt were plotted for these three genes relative to RPS14 for lung, liver, kidney and blood to determine the range of expression variation. Inspection of the graphs suggests that there is a wider range of expression in blood for HTR2B and AGTR1. The extreme variation in expression for expression in blood does not seem to be observed in kidney, liver or lung. These studies need to be extended to additional samples and tissues from phenotyped birds to determine whether the expression variation in blood for AGTR1 extends to other tissues. Current graduate students are investigating SNPs in AGTR1, UTS2D and HTR2B. It will be important to genotype genes associated SNPs and determine whether ascites related alleles are expressing at different levels using the assays and references developed for this dissertation.

Relative expression in humans was computed relative to rpS14 from EST counts from libraries as retrieved from NCBI. The relative expression levels for these human genes compared to chicken genes are presented in Table II.4. Fold expression relative to rpS14 in broilers was computed as 2^(delta Ct) where delta Ct was the average delta Ct for that gene and

tissue for all samples. We used RPS14 as the reference assuming that relative expression in human and chicken are similar (please note that this assumption may not be valid). The expression levels of the three experimental genes are significantly higher in blood since chickens have nucleated red blood cells. However, in liver the chicken appears to express AGTR1 at lower levels than in human. Interestingly, UTS2D and HTR2B are expressed at detectable levels in lung, whereas they were not detected in human EST libraries. Further work on comparing our broiler expression levels to those in human may provide additional insight into differences in tissue level expression, that may contribute to an overall hypertensive condition in broilers.

Table II.1 All qPCR primer pairs with primer name, sequence, Forward (F) and Reverse (R), calculated melting temperature, and annealing temperature used (°C).

PRIMER	SEQUENCE	Adjusted Tm	Annealing temperature
ACE-F	GATCAGTGGCGCTGGAAGGT	63.9	60
ACE-R	CGCCTGGTGGAACTGGAACT	63.9	60
AGTR1-F	TGCCCTTCACTATCTGCATCG	62.4	60
AGTR1-R	TGCATCACTCAGCGTCGAAA	59.8	60
SR-F1	CCTGAACCACCAGAGCCCTTT	64.3	60
SR-R1	TTTGAAAACAAGGTTCTGTGAAAGAG	61.2	60
TBP-F	GCAAAAAGCGAGGAACAGTC	59.8	60
TBP-R	CTGCTGAACTGCTGGTGTGT	61.8	60
Ggrps14F3	CGTGTGACTGGTGGCATGAAGGTGAAGG	72.3	60
Ggrps14R3	CAGAAAGGGTGGTCGCCGTGGACG	72.2	60
RP2B-F	GAGGCGTCAGATCCGTACCAAGT	66.8	60
RP2B-R	CTTGGAAGAGGAGTTTGCAGGCGTAG	69.1	60
UTS2D-F	AGAAGCCAACAGCCCAAACAAGG	65.1	57
UTS2D-R	GGGACGTACACAGTCATGGTCAA	65.1	57

Table II. 2 Comparison of delta Ct values for blood samples for four different housekeeping genes . Birds were from the REL line and classified (Type) as ascites or control based on electrocardiogram. Average Ct values were for triplicate samples and ΔCt values were computed for each reference gene. For each reference gene the average and STD ΔCt value was computed across all samples.

			Average Ct Value				ΔCt Values						
Type	ID	Sex	RPS14	RP2B	TBP	β2M	TBP-RPS14	TBP-RP2B	TBP-β2M	β2M-RPS14	β2M-RP2B	β2M-TBP	RPS14-RP2B
Ascites	16	Male	27.51	28.83	21.84	35.04	-5.7	-7	-13	7.5	6.2	13	-1.3
Ascites	9	Female	24.26	28.01	22.59	32	-1.7	-5.4	-9	7.7	4	9	-3.8
Ascites	21	Male	25.84	28.93	21.84	32.75	-4	-7.1	-11	6.9	3.8	11	-3.1
Ascites	12	Male	29.51	30.23	34.4	33.53	4.9	4.2	1	4	3.3	-1	-0.7
Ascites	8	Male	27.19	27.89	21.22	28.38	-6	-6.7	-7	1.2	0.5	7	-0.7
Ascites	34	Male	26.11	30.36	23.14	39.18	-3	-7.2	-16	13.1	8.8	16	-4.3
Ascites	7	Male	25.2	29.92	22.14	27.94	-3.1	-7.8	-6	2.7	-2	6	-4.7
Ascites	53	Male	25.93	27.29	20.38	30.61	-5.6	-6.9	-10	4.7	3.3	10	-1.4
Ascites	17	Male	25.23	28.71	22.67	35.2	-2.6	-6	-13	10	6.5	13	-3.5
Ascites	57	Male	29.05	30.67	23.05	29.43	-6	-7.6	-6	0.4	-1.2	6	-1.6
Ascites	6976	Male	28.84	29.88	39.03	34.79	10.2	9.2	4	6	4.9	-4	-1
Ascites	6978	Male	28.93	28.94	22.27	33.99	-6.7	-6.7	-12	5.1	5.1	12	0
Control	56	Male	24.47	30.39	22.2	38.44	-2.3	-8.2	-16	14	8.1	16	-5.9
Control	45	Male	26.58	30.53	24.39	35.23	-2.2	-6.1	-11	8.7	4.7	11	-4
Control	51	Male	25.96	27.74	32.57	30	6.6	4.8	3	4	2.3	-3	-1.8
Control	37	Male	25.61	29.84	22.28	35.51	-3.3	-7.6	-13	9.9	5.7	13	-4.2
Control	50	Male	26.74	28.87	34.88	42.37	8.1	6	-7	15.6	13.5	7	-2.1
Control	42	Male	27.15	27.33	19.25	25.53	-7.9	-8.1	-6	-1.6	-1.8	6	-0.2
Control	48	Male	25.4	29.26	22.31	29.83	-3.1	-7	-8	4.4	0.6	8	-3.9
Control	58	Male	27.11	30.16	25.3	33.79	-1.8	-4.9	-8	6.7	3.6	8	-3.1
Control	59	Male	31.54	27.51	21.28	29.22	-10.3	-6.2	-8	-2.3	1.7	8	4
Control	47	Male	25.53	30.65	22.95	40.83	-2.6	-7.7	-18	15.3	10.2	18	-5.1
Control	46	Male	28.53	27.55	32.51	39.52	4	5	-7	11	12	7	1
Control	49	Male	26.44	28.37	33.17	31.85	6.7	4.8	1	5.4	3.5	-1	-1.9
Control	55	Male	22.61	27.66	21.05	29.59	-1.6	-6.6	-9	7	1.9	9	-5.1
Control	43	Male	21.62	25.23	20.44	30.06	-1.2	-4.8	-10	8.4	4.8	10	-3.6
Average							-1.53	-3.91	-8.29	6.76	4.38	8.29	-2.38
STD							5.19	5.47	5.6	4.73	3.9	5.6	2.22

Table II. 3 qPCR analysis for AGTR1 and HTR2B in whole blood using RPS14 and Rp2B (delta Ct value was calculated based on using RPS14 and RP2B as reference genes) . AGTR1 was over expressed in the control birds compared to ascitic birds. AGTR1 with reference genes RPS14 and RP2B was over expressed in ascitic birds compared to control birds.

Type	Gender	SAMPLE	s14-rp2b	agtr1-s14	agtr-rp2b	sert-s14	sert-rp2b
Ascites		19	-12.6	0.1	-12.5	7.5	-5.1
Ascites	Male	57	-2.8	-5.8	-8.6	2.6	-0.2
Ascites	Male	34	-7.1	3.9	-3.3	5.6	-1.6
Ascites	Male	53	-0.7	-1.9	-2.6	3.9	3.2
Ascites	Male	18	-4.0	1.8	-2.1	2.5	-1.5
Ascites	Male	8	-2.9	1.4	-1.5	0.6	-2.4
Ascites	Male	21	1.5	-3.0	-1.5	-0.3	1.2
Ascites	Male	16	0.3	-1.2	-0.9	-1.1	-0.9
Ascites	Male	17	-2.1	1.2	-0.8	-8.3	-10.4
Ascites	Male	7	0.4	-0.8	-0.4	-1.3	-0.9
Ascites	Female	9	-1.5	1.2	-0.3	3.1	1.6
Ascites	Male	20	1.2	-0.9	0.3	-1.6	-0.4
Ascites	Male	12	0.8	-0.4	0.3	0.0	0.8
Control	Male	39	0.8	0.5	1.3	0.2	1.0
Control	Male	56	-2.3	3.8	1.6	0.2	-2.1
Control	Male	45	-2.1	3.8	1.8	0.0	-2.1
Control	Male	51	-1.8	3.7	1.9	0.0	-1.8
Control	Male	37	-2.3	4.5	2.2	1.4	-0.9
Control	Male	50	-3.2	5.5	2.4	1.0	-2.2
Control	Male	42	-2.6	5.6	3.0	1.2	-1.4
Control	Male	48	-3.6	6.5	3.0	-3.1	-6.6
Control	Male	58	-8.5	11.9	3.4	6.8	-1.7
Control	Male	59	-0.8	4.4	3.6	1.4	0.6
Control	Male	47	-2.6	6.3	3.8	5.1	2.5
Control	Male	46	0.1	4.0	4.1	1.2	1.4
Control	Male	49	-5.2	9.4	4.3	2.8	-2.4
Control	Male	55	0.9	3.7	4.6	0.3	1.3
Control	Male	43	0.0	6.0	6.0	3.8	3.8
Rescued	Male	6978	-1.7	8.2	6.5	0.9	-0.8
Rescued	Male	6976	4.1	4.2	8.3	-3.9	0.2

Table II.4 Comparison of expression levels for humans vs. chickens for three candidate genes in four tissues. In the upper panel the EST counts in Genbank are summarized and then utilized to compute expression relative to RPS14. In the lower panel the expression in chicken is computed from average delta Ct values and then computed as expression relative to RPS14. N.D.- not detected; n.d.- not determined.

	Fold relative to S14			EST counts			
	UTS2D	HTR2B	AGTR1	UTS2D	HTR2B	AGTR1	rpS14
Blood	N.D.	1.0	N.D.	0	8	0	8
Kidney	1.0	9.3	8.3	4	37	33	4
Liver	N.D.	N.D.	9.6	0	0	86	9
Lung	N.D.	N.D.	2.5	0	0	5	2

Human UTS2D, SERT, & AGTR1 relative to S14

	Fold relative to S14			EST counts		
	UTS2D	HTR2B	AGTR1	UTS2D	HTR2B	AGTR1
Blood	N.D.	26.0	24.6	0.0	4.7	4.6
Kidney	2.6	3.1	5.9	1.4	1.6	2.6
Liver	4.4	2.2	1.4	2.1	1.1	0.5
Lung	8.6	0.7	1.3	3.1	-0.5	0.3

Chicken UTS2D, SERT, & AGTR1 relative to S14

Fig II. 1 Important candidate genes on chromosome Gga9 & Gga27 (Gga9:13.5‑14.8: AGTR1, and UTS2D, Gga9:16-5HT2B)

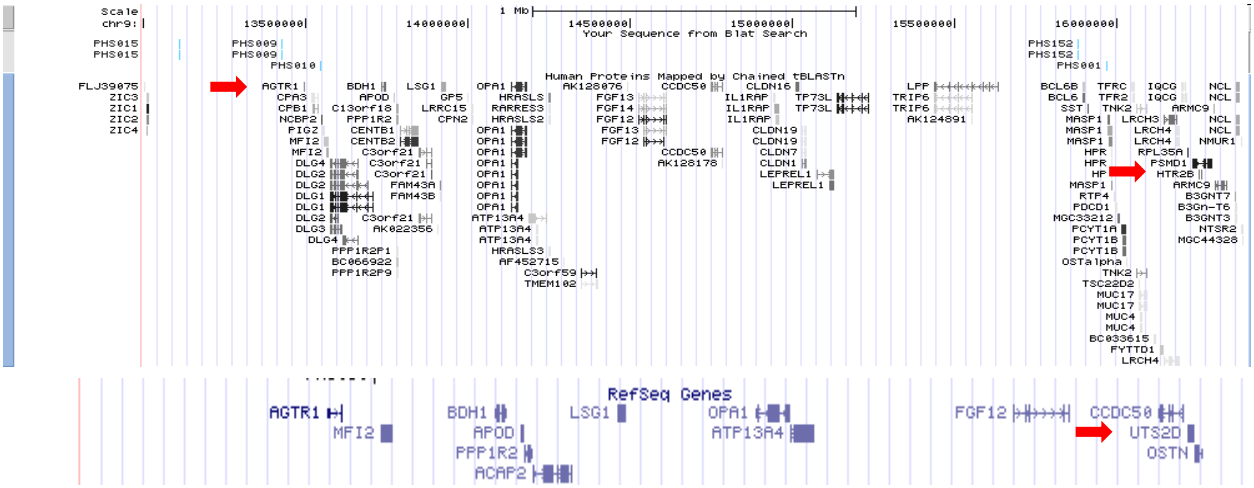


Fig II.2 Genome wide association study for PHS/ascites on Chromosome Gga9 and Gga27.

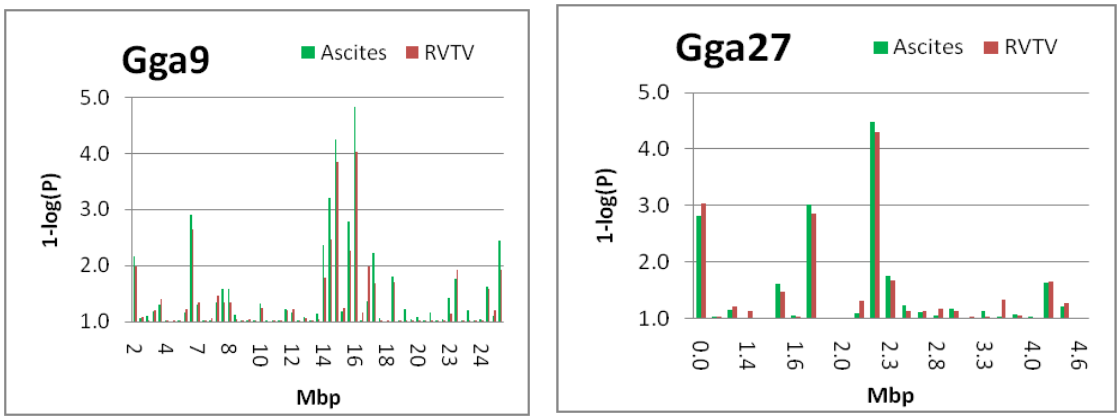
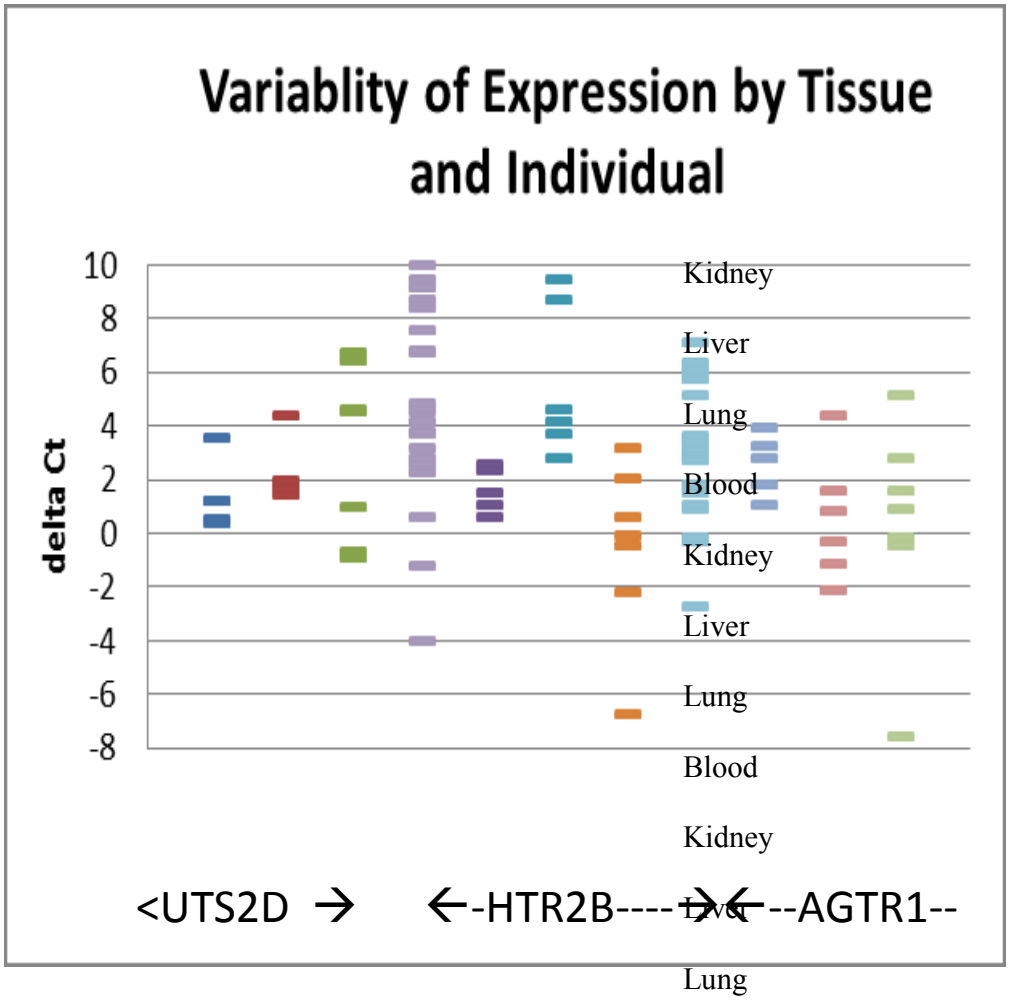


Fig II.3 Analysis of relative expression of three candidate genes in selected tissues from broilers. AGTR1 was over expressed in liver and slightly in lung relative to RPS14. SERT and UTS2D were over expressed in lung relative to RPS14.



III. Chapter three: Initial Molecular Identification of Bacteria Involved in Lameness in Broiler

III.1 Abstract

Lameness is one of the main metabolic diseases related to fast growth in broilers. It is a significant problem in the poultry industry resulting in hundreds of millions of dollars in lost revenue annually. In commercial broilers, the most common cause of lameness is bacterial chondronecrosis with osteomyelitis (BCO). Recently a model has been developed to induce lameness in experimental flocks of broilers. The model includes raising chicken on wire flooring to create footing instability and stress, thereby resulting high incidences of lameness. Our research entails the collection of proximal femoral heads, proximal tibial heads, and blood samples from clinically lame birds, culturing bacteria from these tissues, and PCR sequencing the 16S ribosomal DNA for species identification. This experiment represents the initial molecular identification of the bacteria causing lameness. We initially used chicks that hatched at a commercial hatchery, and were transported to the University of Arkansas poultry research farm. Preliminary work has shown that most cases of lameness in a single floor pen are primarily infected with a single species of bacteria. Surprisingly, the species we identified is not normally associated with poultry. There are two ways through which bacteria can enter the blood stream; either through the respiratory or gastrointestinal tract. We hypothesize that blood-borne bacteria then settle in microfractures in the proximal femoral and tibial heads causing lameness. We found that *Staphylococcus* species are the main species that cause lameness. *Staphylococcus agnetis* represents the main species isolated from the majority of our tissue samples. Knowing the exact bacterial species causing lameness and the route of transmission to broilers will lead to the development of measures for mitigating BCO and ultimately reducing the economic losses.

III.2 Introduction

Lameness in commercial broilers is attributable primarily to bacterial chondronecrosis and osteomyelitis (BCO), which previously was known as femoral head necrosis (Wideman, et al., 2012). BCO is considered the most common cause of lameness and it has been diagnosed worldwide. BCO has been diagnosed in Canada, Europe, Australia, and the United States (Bradshaw, et al., 2002; Dinev, 2009; P. T. McNamee, et al., 1998; Pattison, 1992). The term BCO refers mainly to microbial infection and necrotic degeneration in the proximal head of the femora and tibiae, including the epiphysis, articular cartilage, physis and or growth plate, and the metaphysis. Other rapidly growing bones including the vertebrae also may be affected (Bradshaw et al., 2002; Butterworth, 1999; Carnaghan, 1966; Dinev, 2009; Duff, 1990; McCaskey et al., 1982; McNamee et al., 1998; McNamee and Smyth, 2000; Mutalib et al., 1983; Nairn, 1973; Nairn and Watson, 1972; Riddell, 1992; Thorp, 1994; Thorp and Waddington, 1997; Thorp et al., 1993; Wise, 1971).

Commercial broiler flocks often experience a high incidence of femoral and tibial BCO lesions (Wideman and Prisby, 2013) as shown in fig III.1 and III.2 (Wideman et al., 2012). BCO lesions are mostly observed macroscopically in the proximal femoral and tibial heads. In the absence of the macroscopic lesions of the proximal femoral head, necrotic lesions can be seen on the proximal tibial head near the metaphysis of the growth plate. It is rare to see lesions on the distal ends of the tibiae or femora (Emslie and Nade, 1983; McNamee et al., 1999). Different broiler lines from different countries experience lameness due either proximal femoral or tibial head necrosis or both. Broilers from Holland show a tendency to develop proximal tibial head lesions whereas broilers from Scotland have a high tendency to develop BCO in the proximal

femoral head (Thorp and Waddington, 1997). BCO field outbreaks might affect one leg leaving the contralateral leg macroscopically intact (Dinev, 2009; McNamee et al., 1998).

The pathogenesis of BCO is complex (Butterworth, 1999; P. T. McNamee and Smyth, 2000), but it is generally attributed to the structural immaturity of the growth plate of rapidly growing broilers. Modern broilers weight approximately 40g at hatch and are able to grow to over 4 kg in 8 weeks. If humans were to grow at the same rate, a 3-kg newborn baby would weigh 300 kg after 2 months (Wideman and Prisby, 2013). This high growth rate in broilers causes excessive torque and shear stress to be applied to the immature growth plates of the thoracic vertebrae and the proximal leg bones. This excessive mechanical force causes the formation of clefts and microfractures, often referred to as osteochondrosis, among the physeal and epiphyseal layers of cartilage and columns of chondrocytes. The formation of osteochondrotic clefts causes ischemia and necrosis due to the truncation of the blood vessels that penetrate the columns and layers of cartilage cells. Another cause for local ischemia is thrombosis within the narrow vascular channels, sluggish blood flow that is caused by mechanical compression of the cartilage layers, the inactivity and the resting posture of the fully fed broiler, and the high resistance to blood flow inside the long and narrow metaphyseal vascular channels (Bradshaw et al., 2002; Dinev, 2009; Duff and Randall, 1987; Duff, 1990; Julian, 1985; McCaskey et al., 1982; McNamee et al., 1998; Riddell, 1983, 1992; Thorp, 1988, 1994; Thorp and Duff, 1988; Thorp and Waddington, 1997; Thorp et al., 1993; Wise, 1971). During field outbreaks of BCO most of the lameness develops between 6 and 8 weeks of age (Dinev, 2009; McNamee et al., 1998). The low and sporadic incidence of BCO in research flocks made it difficult to study the etiology, pathogenesis, and the treatment strategies for the disease. In the past, BCO has been produced in broilers and turkeys through the intravenous injection of

Staphylococcus spp. in quantities low enough to avoid septicemia yet sustain bacteremia (Alderson et al., 1986; Carnaghan, 1966; Daum et al., 1990; Emslie and Nade, 1983; Emslie et al., 1983; Griffiths et al., 2008; Mutalib et al., 1983; Nairn, 1973; Smith, 1954). The wire flooring model triggers the development of BCO without the need to inject research flocks with pathogens. The wire flooring system was developed and patented by the University of Arkansas [1]. This model helps maintain a persistent footing instability which results in a sustained mechanical shear and torque stress on vulnerable leg joints. Excessive torque and shear stress are thought to cause BCO by causing micro-trauma, the development of osteochondrosis of the physeal-epiphyseal cartilage, the thrombotic closure of the metaphyseal blood vessels, and finally colonization by translocated bacteria (Duff and Randall, 1987; Emslie et al., 1984; Thorp and Duff, 1988; Duff, 1984; Hocking and Whitehead, 1992; McCaskey et al., 1982; Riddell et al., 1983; Thorp et al., 1993). Bacterial colonization leads to the release of lytic substances which enhance generalized necrosis within the calcifying zone of the metaphysis, thereby destroying the vasculature and removing the struts of the trabecular bone that normally provide structural support for the physeal and epiphyseal cartilages (Emslie et al., 1984; Emslie and Nade, 1983; Wyers et al., 1991). The penetration of physis by bacteria probably through the transphyseal vessels or directly via the epiphyseal vascular complex (evc) leads to hock and hip septic arthritis (Alderson and Nade, 1987; Alderson et al., 1986; Daum et al., 1990; Emslie et al., 1984; Joiner et al., 2005; McNamee et al., 1998; Thorp, 1988).

[1]Provisional patent No.61/499,954 protects the exclusive rights of the University of Arkansas to all uses of the wire flooring technology within the context of evaluating or developing treatments for inducing lameness attributable to osteochondrosis, chondronecrosis ,and osteomyelitis in poultry.

The wire flooring model consistently induces between 20% and 60% lameness attributable to BCO in fast growing broilers. Affected broilers exhibit an obvious, constant and progressive sequence of lesions involving proximal femoral and tibial head necrosis (Wideman et al., 2012).

A wide range of opportunistic bacteria are responsible for causing BCO in broilers. *Staphylococcus aureus* and *E. coli* are predominant and they have been isolated mostly in a mixed culture with other bacteria like *Salmonella* spp. (Andreasen et al., 1993; Butterworth, 1999; Dinev, 2009; Joiner et al., 2005; Kense and Landman, 2011; McNamee et al., 1998; Nairn and Watson, 1972; Tate et al., 1993; Thorp et al., 1993).

In this chapter we will highlight the different kinds of bacteria that we were able to culture and identify, using molecular identification, in samples obtained from broilers raised on wire flooring at the University of Arkansas poultry research farm.

III.3 Materials and methods

III.3.1 Broiler chambers

Broilers used in this experiment were raised at the University of Arkansas poultry research farm with the animal procedures approved by the Arkansas Institutional Animal Care and Use Committee (Protocol # 11002). Experimental chambers and experimental procedures were conducted according to Wideman et al. (2012). Twenty-two pens (10' x 10') were set up in building A364 East on the University of Arkansas Poultry Research Farm. Six pens had wood shavings litter flooring (pens 1, 6, 11, 12, 17 and 22) and the remaining pens had flat wire flooring. Flat wire flooring pens had elevated wire flooring panels. Tube feeders were placed at the front of the pen, and nipples for water supply were placed at the back of the pen to force the

chicks to move back and forth on the wire flooring and thereby help to induce lameness (Wideman et al., 2012).

Broiler chicks used in this experiment were obtained from a commercial hatchery, and were placed at more than 65 chicks per pen at 1 day old of age. The chicks remained unvaccinated. At 14 day age, the population density was reduced to 60 chicks per pen by eliminating the less healthy chicks and runts. This culling procedure was followed on day 14 because necropsies of runts during the first 10 days of age showed macroscopic evidence related to systemic bacterial infection, including osteomyelitis. Birds were allowed a photoperiod of 23 hours light and 1 hour of darkness. Water and feed supply ware provided ad libitum with the feed provided as starter for 1-35 days of age, and finisher for 36-56 days of age. Temperature was adjusted based on chicks' age, and it ranged from 32°C for day 1 to 3, 30°C for day 4 to 6, 28°C for day 7 to10, 26°C for day 11 to 14, and 24°C for day 14-56.

III.3.2 Clinical and post-mortem diagnosis

Beginning at day 14, birds were observed for clinical signs of lameness by walking between the birds inside each pen every 2 days. Birds raised on a wire flooring tend to lie down because of the sustained pressure on their joints, and it is necessary to move them around to detect the earliest signs of lameness. Moving the birds around to look for any apparent gait problems was accomplished using gentle prodding with a broom. Lameness progresses rapidly in broilers and it is very common to see birds, that looked clinically healthy 24-48 hours previously, becoming lame thereafter. Significant increases in cumulative lameness incidences typically develop after 35 days of age, and dramatically increase thereafter because of the rapid increases in body weight (Wideman et al., 2012). The main clinical signs for lame broilers are: difficulties

in standing, obvious limping gait with dipping one or both wing tips, and in the more advance cases, birds become completely immobilized. Birds that show obvious signs of lameness were removed from the flock; blood samples were collected from them, and they were euthanized using CO_2 gas inhalation. Lame birds with BCO die quickly (Dinev, 2009); therefore birds that were found dead were necropsied to look for any proximal femoral or tibial head lesions. Necropsying was done soon after euthanizing the birds, and birds that were found dead were necropsied as soon as they were discovered. The final post-mortem diagnosis was based on the pictures shown in Fig III.1 and III.2, and with reference to the lameness diagnosis chart shown in Fig III.3 (Wideman et al., 2012).

III.3.3 Sample collection and transportation

Blood samples were collected from clinically lame birds using vacutainer tubes (EDTA s anticoagulant). Those birds were then euthanized, and post-mortem lameness diagnosis was conducted. All dissection and cutting utensils were placed in 95% alcohol, and the external skin was sterilized using 95% alcohol. An incision was made through the skin covering the hip and knee joint using a sterile knife. Initial visual diagnoses of the proximal femoral and tibial heads were recorded. Proximal femoral and tibial heads were then collected using a sterile scissor and forceps. Samples were transferred into sterile plastic tubes and placed on ice in a cool box. Samples were then transported to Dr. Douglas Rhoads' laboratory in the Daniel Ferritor building, on the campus of the University of Arkansas.

III.3.4 Bacterial media preparation and sample culturing

As the majority of the literature links *S. aureus*, *E. coli*, and *Salmonella* spp. to lameness in broilers, our initial microbiological investigation for bone and blood samples included

culturing those samples on selective agar and broth media specific for *S. aureus*, *E.coli,* and *Salmonella* spp. The media that we used included mannitol salt agar, Staphylococcus 110, and Staphylococcus broth for *Staphylococcus aureus*, eosin methylene agar and Tryptic soy broth for *E. coli*, and SS agar and Selenite broth for *Salmonella* spp. We used broth media together with agar media for enrichment purposes. Media were prepared according to media manufacturer's instructions, and media were sterilized using an autoclave under 118 or 20 minutes. Agar plates and tubes were stored at 4 °C in a refrigerator.

Samples brought from the farm were cultured on the above mentioned media. Initial findings showed no growth on *E. coli* and *Salmonells* spp. selective media. There was growth on *S. aureus* selective media, but the growth results didn't indicate the presence of *S. aureus*, but rather *Staphylococcus* spp. in general. Based on this result, we decided to switch to using general media that is nutrient agar and nutrient broth, and to use molecular identification for a quick, accurate, and economical identification of the BCO causing microorganisms. Samples were collected with a sterile cotton swab rubbed on the bone lesion. The swab is then streaked onto nutrient agar plates and cultured for 48 hours. Quadrant streaking technique for a single colony was used when culturing the samples. Agar plates were incubated in an incubator for 48 hours at 37°C, and broth tubes were incubated in a shaker incubator for aeration for 48 hours at 37°C.

III.3.5 DNA Extraction

Single colonies grown nutrient agar plates were picked using a sterile tooth pick and transferred into sterile microfuge tubes that contain 40µl of sterile water. Microfuge tubes were allowed to sit in a 90 °C water bath for 8-10 minutes. Samples were then spun using a microfuge at 10 K for 2-3 minutes. Samples were then stored at -20°C.

III.3.6 Polymerase Chain Reaction and gel electrophoresis

The 16S ribosomal DNA was amplified using previously published broad-range bacterial primers, Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3') (1) and Bact-1391R (5'-GACGGGCGGTGTGTRCA-3') (Eckburg et al., 2005). Forty microliter Polymerase chain reaction was conducted using 96 well plates with either an Eppendorf Mastercycler gradient or an MJ Research PTC-100 thermocycler using the following: 4 µl of 10X buffer, 0.4 µl of 20 mMdNTP, 0.6 µl of 20U Taq polymerase, 4 µl of bacterial DNA, 0.8 µl of 50µM forward and reverse primers, and 30.2 µl of diH2O. The following PCR conditions were used: 90°C for 30seconds for the initial denaturation followed by 30 cycles of 90°C denaturation for 15 seconds, 30 seconds annealing at 68 °C, 72°C elongation for 90 seconds, and final elongation for 3 minutes at 72°C. PCR products (4µl) were resolved on standard 1.5% agarose gels to assess quality of the PCR.

III.3.7 Gel scanning

A Typhoon model 9600 or FLA9500 (GE Health Care) were used to scan gels, and gel images were analyzed using ImageQuant software.

III.3.8 Sample cleaning and preparation for sequencing

PCR product was cleaned using RapidTips (from Diffinity Genomics)), and quantified using Hoefer Fluorometer TKO 100. Product was mixed with the forward primer, and submitted for sequencing at the DNA Resource Center, Center of Excellence for Poultry Science, University of Arkansas.

III.3.9 Sequence analysis

Sequences were assembled using SeqMan Pro (DNASTAR) and were then trimmed to the common region (typically 700 bp). Final alignment and phylogenetic tree were conducted using MegAlign (CustalV) with Reference 16S rDNA from NCBI. NCBI blast tool, and Ribosomal Database Project (RDP) sequence match tool were used to identify the single species in each sample.

III.4 Results

For the purpose of identifying the bacteria that causes BCO, we collected blood and bone samples from 24 lame broilers that were raised on wire flooring. Samples included 12 females (50%) and 12 males (50%). Fifteen broiler (62.5%) were from line Y, and 9 (37.5%) were from line Z. Samples were collected from lame, completely immobilized birds with ages between 48 and 53 days. No samples were collected from clinically healthy birds in this initial experiment. Post-mortem diagnosis revealed the presence of typical proximal femoral and tibial head lesions. Out of the total 96 collected bone samples (48 proximal femora, 48 proximal tibiae), there were a total of 24 right femoral head samples, and 24 left femoral head samples. Eleven right femoral heads (45% of the right femoral heads) were diagnosed as Femoral Head Transitional degeneration (FHT), 9 (37.5%) were diagnosed as Femoral Head Necrosis (FHN), 2 (8.3%) were diagnosed as Femoral Head Separation (FHS), and 2 were diagnosed as being macroscopically normal. Eleven left femoral heads (45.8% of the left femoral heads) were diagnosed as FHN, 7 (29.2%) were diagnosed as FHT, & 4 (16.7%) were diagnosed as FHS, and 2 were diagnosed as being macroscopically normal (Table III.1).

There were a total of 24 right tibial heads and 24 left tibial heads. Ten of the right tibial heads (41%) were diagnosed as Severe Tibial Head Necrosis (THNs), 8 (33.3%) were diagnosed as Caseous Tibial Head Necrosis (THNc), 4 (16.7%) were diagnosed as Tibial Head Necrosis (THN), and 2 (8.3%) were diagnosed as being macroscopically normal. Eight (33.3% of the left tibial heads) were diagnosed as THNs, 8 (33.3%) were diagnosed as THNc, 5 (20.8%) were diagnosed as THN, and 3 (12.5%) were diagnosed as being macroscopically normal (Table III.2).

The final total number of analyzed blood and bone samples was 93. This excludes the samples that didn't show bacterial growth (n = 5 blood samples, n = 9 femoral and tibial samples) and those with bad sequences (n = 3 blood samples; n = 10 femoral and tibial samples). Out of the 93 analyzed samples, there were 22 (23.7%) right tibial head samples, 21 (22.6%) left tibial head samples, 17 (18.3%) right femoral heads, 17 (18.3%) left femoral heads, and 16 (17.2% of the total samples) blood samples (Table III.3). The final results showed the presence of homo-infection, mixed species, and mixed genera. Ten males (83.33%) showed a homo-infection, 1 male (8.33%) showed a mixed species infection, and 1 male (8.33%) showed mixed genera. On the other side, 6 females (50%) showed a homo-infection, and 6 females (50%) showed a mixed species infection (Table III.4). Final sequence data revealed the presence of 7 different bacterial species and genera. *Staphylococcus agnetis* was identified in 81 samples (87.1%) out of the total 93 assembled sequences followed by *S. aureus* which was identified in 3 samples (3.23%), *Staphylococcus hominis* in 3 samples (3.23%), *Staphylococcus epidermidis* in 2 samples (2.15%), *Staphylococcus xylos*us in 2 samples (2.15%), and *Enterococcus faecalis* in 1 sample (1.08%) (Table III.5).

Bacteria identified were different based on tissue location. *S. agnetis* was identified in 21 right tibial heads, 20 left tibial heads, 15 blood, 13 right femoral heads, and 12 femoral head samples. *S. aureus* was identified in 1 right femoral head, 1 left femoral head, and 1 blood sample. *S. hominis* was identified in 1 right femoral head, 1 left femoral head , and 1 left tibial head sample. *S. epide*rmidis was identified in 1 right femoral head, and 1 left femoral head sample. *S. xylosus* was identified in 1 right femoral head and 1 left femoral head sample. *E. faecalis* was identified in 1 right tibial head sample. *S. saprophyticus* was identified in 1 left femoral head sample (Table III.6).

There were minimal line differences as far as bacterial species diagnosed. *S. agnetis* was identified in 43 samples from line Y and 38 samples from line Z. *S. aureus* was identified in 2 samples from line Y and 1 sample from line Z. *S. epidermidis* was identified in 2 samples from line Y only. *S. hominis* was identified in 3 samples from line Y only. *S. saprophyticus* was identified in 1 sample from line Z only. *S. xylosus* was identified in 2 samples from line Y only, *E. faecalis* was identified in 1 sample from line Y (Table III.7).

Bacterial species tended to differ minimally based on broiler gender. *E. faecalis* was identified in 1 sample taken from a male broiler. *S. agnetis* was diagnosed in 41 male samples and 40 female samples. *S. aureus* was identified in 1 male sample and 2 female samples. *S. epidermidis* was identified in 1 male sample and 1 female sample. *S. hominis* was identified in 2 male samples, and 1 female samples. *S. saprophyticus* was identified in 1 female sample only. *S. xylosus* was identified in 2 female samples only (Table III.8).

The types of bacteria identified in different bone samples differed. *E. faecalis* was identified in 1 sample with a THNc. *S. agnetis* was identified in 12 samples with FHT, 10

samples with FHN, 1 sample with FHS, 9 samples with THN, 16 samples with THNs, 15 samples with THNc, and was isolated from 3 of the the femoral and tibial samples that were diagnosed during necropsy as being macroscopically normal in appearance. *S. aureus* was identified in 2 samples with FHT. *S. epidermidis* was identified in 1 sample with FHT and 1 sample with FHS. *S. hominis* was identified in 1 sample with FHT, 1 sample with FHN, and 1 sample with THNs. *S. saprophyticus* was identified in 1 normal sample. *S. xylosus* was identified in 2 samples with FHT (Table III.9).

III.5 Discussion

Multiple opportunistic pathogenic bacteria have been isolated from BCO lesions, including *S. aureus*, *Staphylococcus* spp., *E. coli*, and *Enterococcus cecorum* (Andreasen et al., 1993; Butterworth, 1999; Dinev, 2009; Joiner et al., 2005; Kense and Landman, 2011; McNamee, 1998; Nairn and Watson, 1972; Smeltzer and Gillaspy, 2000; Stalker et al., 2010; Tate et al., 1993; Thorp et al., 1993). Our experimental results show that BCO involves a range of bacterial species. *Staphylococcus* spp. constitute the main species identified in our bone and blood samples. *S. agnetis* was identified in 81 (87%) out of the 93 analyzed samples for this experiment. *S. agnetis* is Gram positive, non-motile, non-spore forming, and coagulase negative species that is known for causing bovine subclinical and mild mastitis (Taponen et al., 2012). It is not that surprising to find that *Staphylococcus* spp. represents the majority of diagnosed bacterial species. However, it is surprising to see a mastitis causing bacteria, *S. agnetis*, can cause lameness in broilers. There is more than one route by which of *S. agnetis* can be transmitted from cows to broiler. For instance, *S. agnetis* might be transmitted via flies, feed, farm crew, birds, or air. There was little difference between genders and lines as far as the frequency with which *S. agnetis* was isolated from bone samples. Multiple examples of mixed

infection in different infection sites were demonstrated. Some bacterial species identified in this experiment previously had been identified in lame broilers. For example, *S. xylosus* was isolated from 10.5 % of lame broilers in a study that was carried out by McNamee (McNamee, 1998). The different bacterial species identified in this experiment were found in all 5 different tissue samples collected from lame broilers. *S. agnetis* was found in all tissue samples, but the majority of *S. agnetis* was found in proximal right and left tibial heads which might be attributed to the fact that proximal tibial head lesions represented the main cause for lameness in these broilers.

Other bacterial species identified in this experiment were found mainly in right and left femoral heads. We have identified a more diverse bacterial species in samples taken from broilers belong to line Y and specifically from proximal femoral head samples. Line Y developed a higher lameness incidence than other line used in this experiment. The higher susceptibility of broilers from line Y to lameness might be the reason behind a more diverse bacterial species. Broiler chicks used for this experiment were obtained from a commercial hatchery, so the broiler chick source will change in the subsequent experiment, to see if chick sources affect the microbiological outcome. Also, finding the main source of transmission of lameness causing bacteria will help to prevent or limit transmission of such kinds of bacterial species and ultimately may help to reduce the lameness incidence.

Fig III.1 Stages of proximal femoral head degeneration leading progressively to bacterial chondronecrosis with osteomyelitis (BCO): 1, normal proximal femoral head; 2, femoral head separation (FHS: epiphyseolysis); 3–5, progressive necrosis, ulceration, erosion, and fracturing of the growth plate (femoral head transitional degeneration, FHT); 6–8, perforation, fracturing, and necrosis/osteomyelitis of the femoral head (femoral head necrosis, FHN) (Wideman et al., 2012).

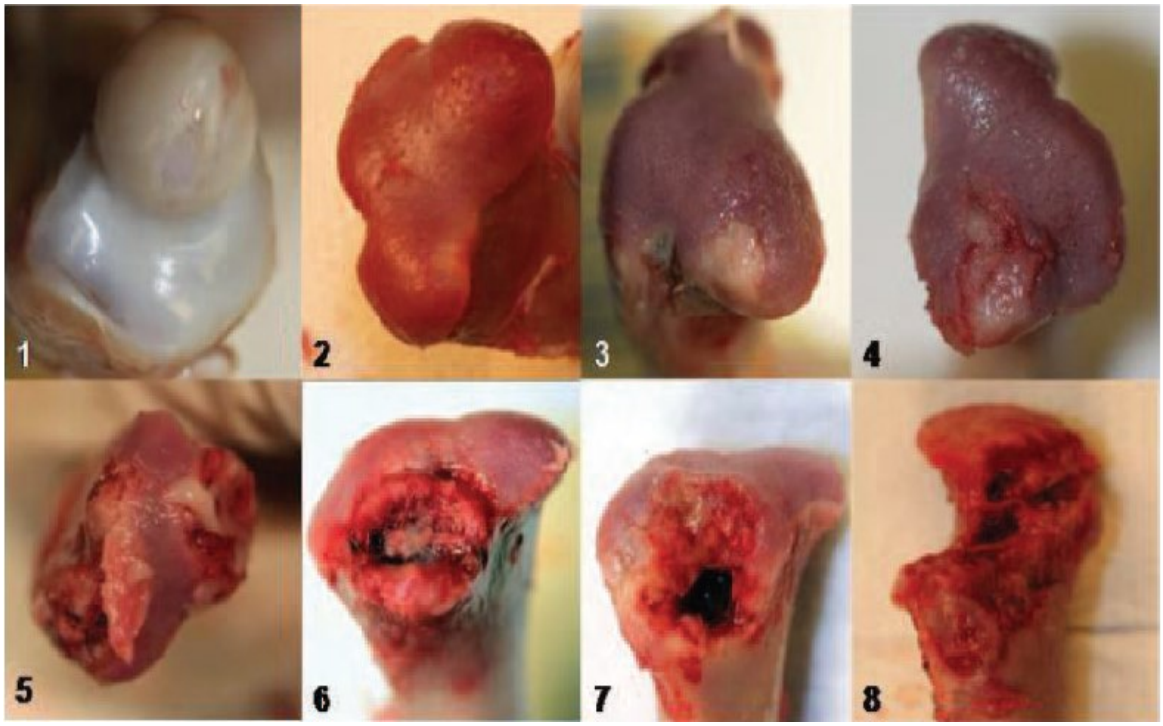


Fig III.2 Stages of proximal tibial head necrosis (THN) leading progressively to bacterial chondronecrosis with osteomyelitis (BCO): 1, normal proximal tibial head with struts of trabecular bone in the metaphyseal zone fully supporting the growth plate; 2–5, necrotic voids (N) in the metaphyseal zone undermine the support of the growth plate, leading to microfractures of the growth plate. Necrotic voids typically were filled with fibrinonecrotic exudate. Lytic channels (small arrows) penetrate from the necrotic voids into the growth plate. Bacterial infiltration and sequestrae (open arrows) provide macroscopic evidence of osteomyelitis. Sequestrae occasionally can be seen in the secondary ossification center of the epiphysis (upper open arrow, stage 7). Necrotic voids can communicate with precocious ectopic extensions of the marrow cavity (M).

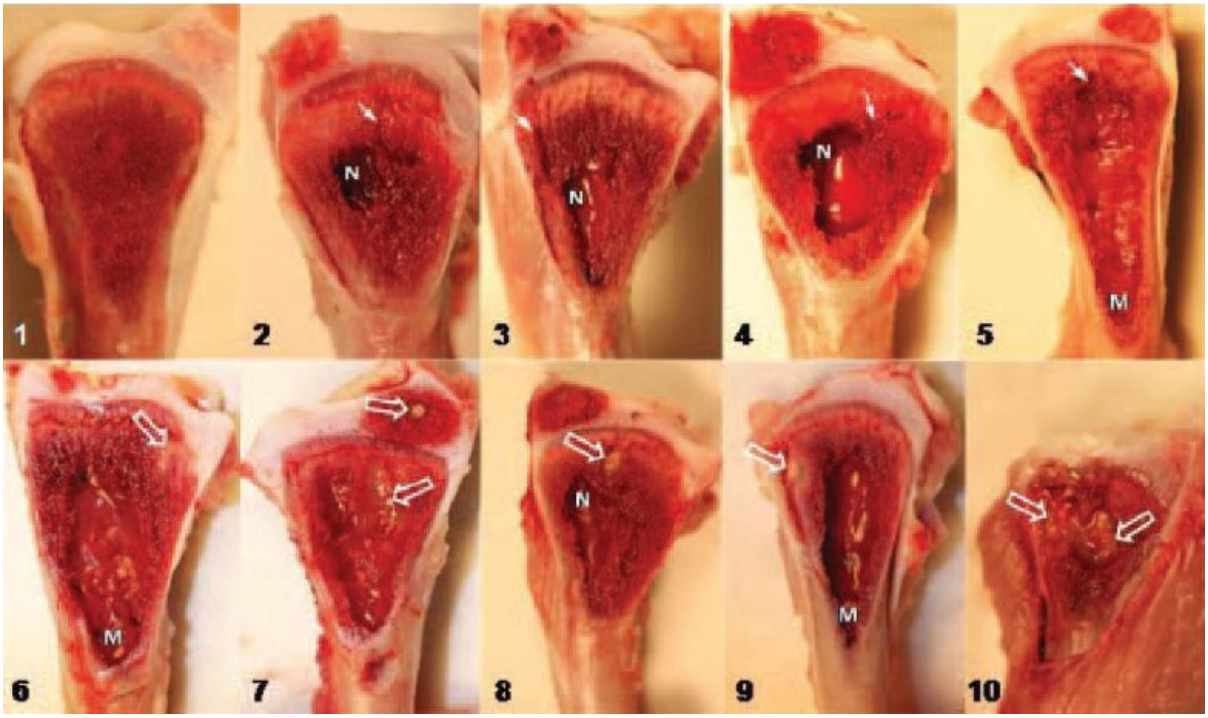


Fig III.3 Lameness diagnosis sequence (Wideman 2011)

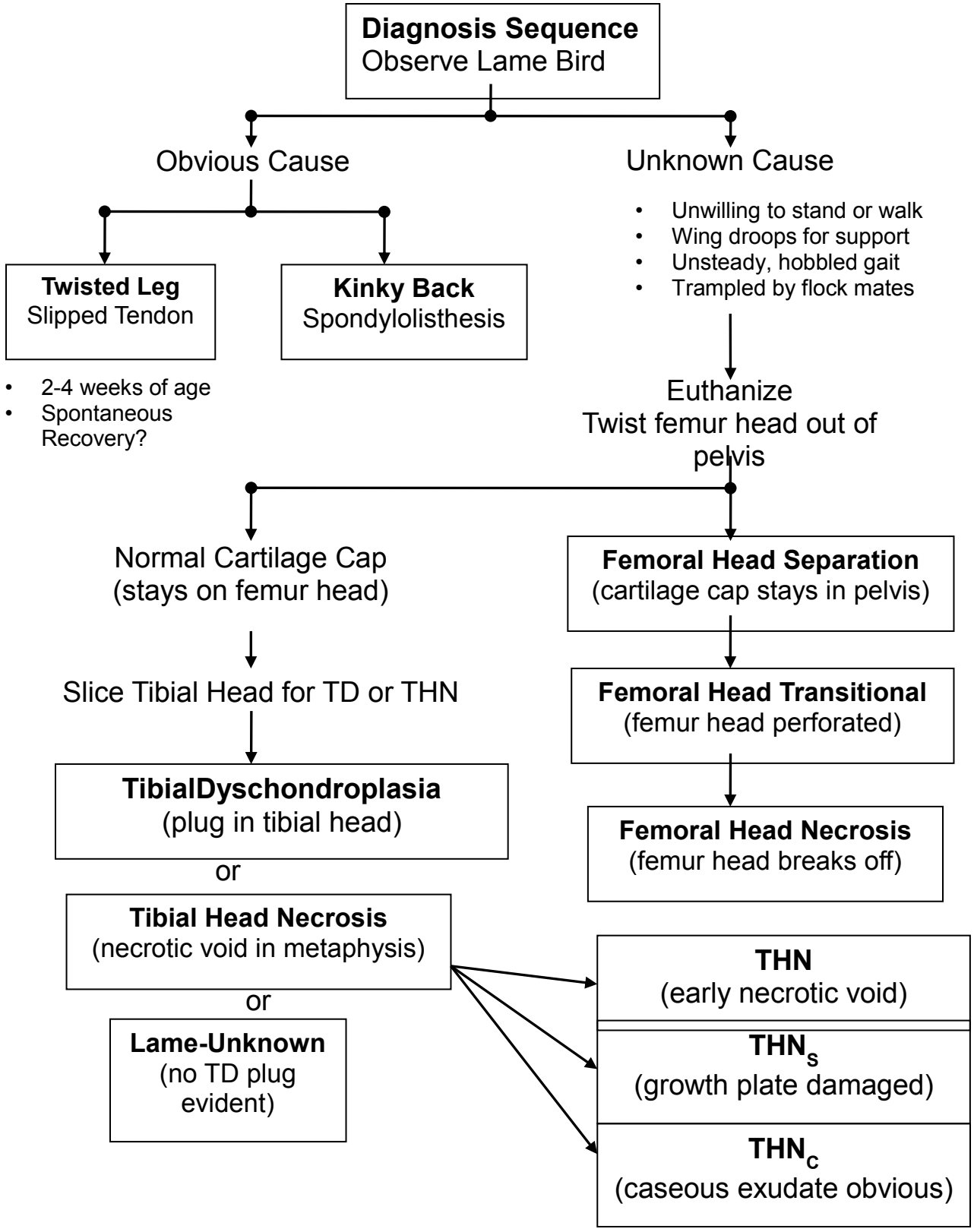


Table III.1 Proximal right and left femoral head (RF & LF) lesions for samples collected from clinically lame broilers in BCO experiment 1. Bone lesion categories include: Femoral Head Separation (FHS), Femoral Head Transitional (FHT), Femoral Head Necrosis (FHN), and Macroscopically Normal.

Femoral Diagnostic Category	RF	%	LF	%
FHS	2.0	8.3	4	16.7
FHT	11.0	45.8	11	45.8
FHN	9.0	37.5	7	29.2
Macroscopically Normal	2.0	8.3	2	8.3
Total	24.0	100.0	24	100.0

Table III.2 Proximal right and left tibial (RT & LT) head lesions for samples collected from clinically lame broilers in BCO experiment 1. Bone lesion categories include: Tibial Head Necrosis (THN), severe Tibial Head Necrosis (THNs), caseous Tibial Head Necrosis (THNc), and Macroscopically Normal.

Type of infection	RT	%	LT	%
THN	4.0	16.7	5	20.8
THNs	10.0	41.7	8	33.3
THNc	8.0	33.3	8	33.3
Macroscopically Normal	2.0	8.3	3	12.5
Total	24.0	100.0	24	100.0

Table III.3 Total number of successfully analyzed tissue samples (out of 24 collected per category) in BCO experiment 1, and percentage of samples analyzed by tissue type. Tissue samples include: Blood, Right Femur (RF), Left Femur (LF), Right Tibia (RT), and Left Tibia (LT).

Type of tissue sample	Number of analyzed samples	%
Blood	16	17.2
RF	17	18.3
LF	17	18.3
RT	22	23.7
LT	21	22.6
Total	93	100.0

Table III.4 Type of infection based on bird's gender in BCO experiment 1

Infection	Male	%	Female	%
Homogenous	10	83.33	6	50
Mixed Species	1	8.33	6	50
Mixed Genera	1	8.33	0	0
Total	12	100	12	100

Table III.5 Types of bacterial species identified in tissue samples in BCO experiment 1

Bacterial species	Total number	%
Enterococcus faecalis	1.00	1.08
Staphylococcus agnetis	81.00	87.10
Staphylococcus aureus	3.00	3.23
Staphylococcus epidirmidis	2.00	2.15
Staphylococcus hominis	3.00	3.23
Staphylococcus saprophyticus	1.00	1.08
Staphylococcus xylosus	2.00	2.15
Total	93.00	100.00

Table III.6 Types of bacterial species identified based on tissue in BCO experiment 1. Tissue samples include: Blood, Right Femur (RF), Left Femur (LF), Right Tibia (RT), and Left Tibia (LT).

Bacteria	RF	LF	RT	LT	BL	Total
Enterococcus faecalis	0	0	1	0	0	1
Staphylococcus agnetis	13	12	21	20	15	81
Staphylococcus aureus	1	1	0	0	1	3
Staphylococcus epidirmidis	1	1	0	0	0	2
Staphylococcus hominis	1	1	0	1	0	3
Staphylococcus saprophyticus	0	1	0	0	0	1
Staphylococcus xylosus	1	1	0	0	0	2
Total	17	17	22	21	16	93

Table III.7 Bacterial distribution based on broiler line in BCO experiment 1

Bacterial species	Y	Z	Total
Enterococcus faecalis	1	0	1
Staphylococcus agnetis	43	38	81
Staphylococcus aureus	2	1	3
Staphylococcus epidirmidis	2	0	2
Staphylococcus hominis	3	0	3
Staphylococcus saprophyticus	0	1	1
Staphylococcus xylosus	2	0	2
Total	53	40	93

Table III.8 Bacterial distribution based on broiler gender in BCO experiment 1

Bacteria	Male	Female	Total
Enterococcus faecalis	1	0	1
Staphylococcus agnetis	41	40	81
Staphylococcus aureus	1	2	3
Staphylococcus epidirmidis	1	1	2
Staphylococcus hominis	2	1	3
Staphylococcus saprophyticus	0	1	1
Staphylococcus xylosus	0	2	2
Total	46	47	93

Table III.9 Bacterial distribution based on bone lesion in BCO experiment 1. Bone lesion categories include: Tibial Head Necrosis (THN), severe Tibial Head Necrosis (THNs), caseous Tibial Head Necrosis (THNc), and Macroscopically Normal(Nor) .

Bacteria	FHT	FHN	FHS	THN	THNs	THNc	Nor	Total
Enterococcus faecalis	0	0	0	0	0	1	0	1
Staphylococcus agnetis	12	10	1	9	16	15	3	66
Staphylococcus aureus	2	0	0	0	0	0	0	2
Staphylococcus epidirmidis	1	0	1	0	0	0	0	2
Staphylococcus hominis	1	1	0	0	1	0	0	3
Staphylococcus saprophyticus	0	0	0	0	0	0	1	1
Staphylococcus xylosus	2	0	0	0	0	0	0	2
Total	18	11	2	9	17	16	4	77

IV. Chapter four: Further molecular investigation of bacteria causing lameness in broiler

IV.1 Introduction

To further investigate the bacteria causing lameness in broilers, an experiment was designed to follow up on the initial findings from the previous experiment. Broiler chicks were obtained from the University of Arkansas Poultry Research Hatchery to see if changing the hatchery might affect the bacterial species isolated from lame birds. Moreover, the blood of healthy birds was tested to see if bacteria can be isolated from the blood of clinically healthy birds. Samples also included right and left femoral and tibial heads, and blood samples from clinically lame birds. Our findings in this experiment matched the findings from experiment 1.

S. agnetis was the main organism recovered from the majority of the samples in this experiment. *S. agnetis* was also recovered from the blood of clinically healthy birds. We have found that changing the hatchery has no effect of the bacteria recovered from lame birds.

IV.2 Materials and methods

IV.2.1 Broiler chambers

Chicks used in this experiment were obtained from University of Arkansas Poultry Research Hatchery. The animal procedures approved by the Arkansas Institutional Animal Care and Use Committee (Protocol # 11002). Experimental chambers and experimental procedure was conducted according to Wideman et al. (2012). Eight pens had a wood shavings litter flooring (Control pens) and 16 pens had a flat wire flooring (Leg Challenge pens) in building A364 East on the University of Arkansas Poultry Research Farm. Birds in leg challenge pens were raised on elevated wire flooring panels. Tube feeders were placed at the front of the pen,

and nipples for water supply were placed at the back of the pen to force the chicks to move back and forth on the wire flooring to induce lameness (Wideman et al., 2012).

Seventy unvaccinated chicks were placed per pen at 1 day old of age. At 14 day age, the total number was reduced to 60 chicks per pen by eliminating all runts. Runts were removed at day 14 because necropsying runts during the first 10 days of age showed macroscopic evidence related to systemic bacterial infection including osteomyelitis. Birds were allowed a photoperiod of 23 hours light and 1 hour of darkness. Water and feed were provided ad libitum with the feed provided as starter for days 1-35, and finisher for days 36-56. Temperature was adjusted based on chicks' age, and it ranged from 32°C for day 1 to 3, 30°C for day 4 to 6, 28°C for day 7 to10, 26°C for day 11 to 14, and 24°C for day 14-56.

IV.2.2 Clinical and post-mortem diagnosis

Beginning at day 14, birds were observed for clinical signs of lameness by walking between the birds inside each pen every 2 days. Birds raised on a wire flooring tend to lie down, and it is necessary to move them around to be able to see whether or not they are lame. Moving the birds around was facilitated with gentle pressure applied with a broom. Lameness progresses rapidly in broilers and it is very common to see birds that looked clinically healthy 24-48 hours previously, becoming lame thereafter. Lameness typically starts after 35 d of age, and increases dramatically because of the fast increase in body weight (Wideman et al., 2012). The main clinical signs for lame broilers are: difficulties in standing, an obvious limping gait with dipping of one or both wing tips, and in the more advance cases, birds become completely immobilized. Birds that show obvious signs of lameness were removed from the flock; blood samples were collected from them, and they were euthanized using CO_2 gas inhalation. Lame birds with BCO

die quickly (Dinev, 2009); therefore birds that were found dead were necropsied to look for any proximal femoral or tibial head lesions. Necropsying was done soon after euthanizing the birds, and birds that found dead were necropsied soon after they were seen. The final post-mortem diagnosis had been done by referring to Dr. Wideman's lameness diagnosis chart Fig III. 3, and post-mortem diagnosis pictures shown in Fig III.1 and III.2. (Wideman et al., 2012).

IV.2.3 Sample collection and transportation

In this experiment the total number of clinically lame? birds sampled was increased to 27 birds, and blood samples also were taken from apparently healthy birds. Blood samples were collected from 40 clinically healthy birds at age 35 days using vacutainer tubes with a manufacturer's ethylenediaminetetraacetic acid (EDTA) anticoagulant. An additional five samples, 4 bone samples and one blood sample were then collected from each clinically lame bird. Blood was collected first, then the birds were euthanized and a post-mortem diagnosis was conducted. All dissection and cutting utensils were placed in 95% alcohol, and the external skin was sterilized using 95% alcohol. An incision was made through the skin covering the hip and knee joint using a sterile knife. Initial visual diagnoses of the proximal femoral and tibial heads were recorded. Proximal femoral and tibial heads were then collected using a sterile scissors and forceps. Samples were transferred into sterile plastic tubes and placed on ice in a cool box. Samples were then transported to Dr. Douglas Rhoads' molecular biology laboratory located inside the Daniel Ferritor building, on the campus of the University of Arkansas.

IV.2.4 Bacterial media preparation and sample culturing

We have found that Tryptic soy agar and broth to be very effective in growing bacteria from bone or blood samples. Therefore, each sample was inoculated on nutrient broth, nutrient agar, tryptic soy broth, and tryptic soy agar. Quadrant streaking technique was used to inoculate samples on agar plates to obtain single colonies. Agar plates were incubated in an incubator for 48 hours at 37°C, and broth tubes were incubated in a shaker incubator for aeration for 48 hours at 37°C.

IV.2.5 DNA Extraction

To produce crude DNA for PCR we used a sterile 96 well PCR plate. We added 40 microliter of sterile water in each well. Each streak on the master plate is then sampled by barely touching the streak with the end of a sterile, flat (not pointed) toothpick. The toothpick is then transferred to a well on the plate. Once all the wells have been inoculated, we twirled each toothpick between the index finger and thumb to dislodge the bacteria and throw away the toothpick. We then sealed the PCR plate with tape and put the plate in a PCR machine programmed for 10 minutes at 100°C (Boiling) then 20°C for 10 sec. After the heat treatment, we spun the plate at 500xg. Samples were then stored at -20°C freezer. Samples were tested using PCR and gel electrophoresis, and the final results suggested the effectiveness of this simple DNA extraction technique.

IV.2.6 Polymerase Chain Reaction and gel electrophoresis

The 16S ribosomal DNA was amplified using previously published broad-range bacterial primers, Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3') (1) and Bact-1391R (5'-GACGGGCGGTGTGTRCA-3') (Eckburg, et al., 2005). Forty microliter Polymerase chain

reaction was conducted using 96 well plates with either an Eppendorf Mastercycler gradient or an MJ Research PTC-100 thermocycler using the following: 4 µl of 10X buffer, 0.4 µl of 20 mMdNTP, 0.6 µl of 20U Taq polymerase, 4 µl of bacterial DNA, 0.8 µl of 50µM forward and reverse primers, and 30.2 µl of diH2O. The following PCR conditions were used: 90°C for 30seconds for the initial denaturation followed by 30 cycles of 90°C denaturation for 15 seconds, 30 seconds annealing at 68 °C, 72°C elongation for 90 seconds, and final elongation for 3 minutes at 72°C.

PCR products were resolved using 1% agarose gels. Four microliters of the PCR product was mixed with 4 µl of the loading dye, which can be prepared by mixing 1 ml of the ficol with 1 µl of ethidium bromide, and we used PGEM5 as a marker. Samples were mixed very well with the loading dye, and were pipetted into the agarose gel wells. Samples were allowed to run for 90 minutes at 70 volts/hour.

IV.2.7 Gel scanning:

A Typhoon imager, model 9600 from GE Health Care, and FLA9600were used to scan gels, and gel images were analyzed using ImageQuant software.

IV.2.8 Sample cleaning and preparation for sequencing

PCR product was cleaned using RapidTips (from Diffinity Genomics) technique. This cleaning technique is an effective technique because it is characterized by: excellent yield, time saving, cost effectiveness, easy to use, and is environmentally friendly technique. Samples then quantified using Hoefer Fluorometer TKO 100 machine. Primers and water then added, and samples were sent for sequencing at the DNA Resource Center, Center of Excellence for Poultry Science, University of Arkansas. Samples were sent for sequencing in a standard 96 wells plate with the DNA sequence request form which was obtained from the DNA resource center.

IV.2.9 Sequence analysis

Sequences assembled using SeqMan Pro (DNASTAR) and trimmed to common region. Final alignment and phylogenetic tree using MegAlign (CustalV) with Reference 16S rDNA from NCBI. NCBI blast tool, and Ribosomal Database Project (RDP) sequence match tool were used to identify the single species in each sample.

IV.3 Results

We collected bone and blood samples from 27 lame broiler chickens. The lame broilers sampled included 8 females (29.6%) and 19 males (70.4%). Eight broilers (29.6%) were from line Y, 7 (25.9%) were from line Z, 7 (25.9%) were from line W, and 5 (18.5%) were from line X. Samples were collected from lame completely immobilized birds between 12 and 52 days of age. Post-mortem diagnosis revealed the presence of typical proximal and tibial head lesions. There were 27 right femoral head samples, and 27 left femoral head samples. Seven (25.9%) right femoral heads were diagnosed as macroscopically normal, eleven right femoral heads (45.7% of the right femoral heads) were diagnosed as FHS, 5 (18.5%) were diagnosed as FHT, and 4 (14.8%) were diagnosed as FHN. Eleven left femoral heads (40.7 % of the left femoral heads) were diagnosed as FHN, 8 (29.6%) were diagnosed as FHS, 5 (18.5 %) were diagnosed as FHT, and 3 (11.1% were diagnosed as macroscopically normal (Table IV.1).

There were 27 right tibial heads and 27 left tibial heads. Seventeen (63.1% of the right tibial heads) right tibial heads were diagnosed as THN, 5 (18.5 %) were diagnosed as macroscopically normal, 3 (11.1%) were diagnosed as THNc, and 2 (7.4%) were diagnosed as THNs. Twelve (44.4%) of the left tibial heads were diagnosed as THN, 8 (29.6 %) were

diagnosed as THNs, 4 (14.8 %) were diagnosed as macroscopically normal, and 3 (11.1%) were diagnosed as THNc (Table IV.2).

The final total number of analyzed samples was 74 samples excluding the samples that didn't show bacterial growth (n = 12 blood samples; n = 12 femoral and tibial samples) and those with bad sequences (n = 7 blood samples; n = 30 femoral and tibial samples). Out of the 74 analyzed samples, there were 22 (29.7%) left tibial head samples, 15 (20.3%) right tibial head samples, 15 (20.3%) right femoral heads, 14 (18.9%) left femoral heads, and 8 (10.3%) blood samples (Table IV.3). The final results showed the presence of homo-infection, mixed species, and mixed genera. Nine males (47.4% of the total number of males) showed a homo-infection, 7 males (36.8%) showed a mixed species infection, and 3 males (15.8%) showed mixed genera. For the females, 5 (62.5%) showed a homo-infection, 3 (37.5%) showed a mixed species infection, and there was no mixed genera infection in females (Table IV.4).

Final sequence data revealed the presence of 12 different bacterial species and genera. *S. agnetis* was identified in 50 samples (67.6 %) out of the total 74 assembled sequences followed by *S. xylosus* which was identified in 10 samples (13.5%), *S. cohnii* in 2 samples (2.7%), *S. aureus* was identified in 2 samples (2.7%). *S. hominis* was identified in 2 samples (2.7%). *Enterococcus faecalis* in 2 samples (2.7%), *S. saprophyticus* in 1 samples (1.4 %), *Enterococcus hirae* in 1 sample (1.4%), *Enterococcus casseliflavus* in 1 sample (1.4%), *Kurthiagibsonii* in 1 sample (1.4%), *Enterococcus faecium* in 1 sample (1.4%), and *Staphylococcus* spp. in 1 sample (1.4%) (Table IV.5).

Bacteria identified were different based on tissue location. *S. agnetis* was identified in 16 left tibial heads, 14 right tibial heads, 8 left femoral heads, 7 blood samples, and 5 right femoral

heads. *S. xylosus* was identified in 3 right and 3 left femoral heads, 3 left tibial heads, and 1 right tibial heads. *S. cohnii* was identified in right and 1 left femoral heads. *S. aureus* was identified in 1 right femoral head and 1 left tibial head. *S. hominis* was identified in 1 right and 1 left tibial heads. *Enterococcus faecalis* in 1 right femoral head and 1 blood sample, *S. saprophyticus* in 1 left tibial head sample, *Enterococcus hirae* in 1 right femoral head, *Enterococcus casseliflavus* in 1 left femoral head sample, *Kurthia gibsonii* in 1 right femoral head sample, *Enterococcus faecium* in 1 right femoral head sample, and *Staphylococcus* spp. in 1 left tibial head sample (Table IV.6).

S. agnetis was identified in 18 samples that were taken from birds belong to line W, 13 samples from line Z, 11 samples from line X, and 8 samples from Y. *S. xylosus* was identified in 5 samples from line Y, 3 from line Z, and 2 from line X. *S. cohnii*was identified in 1 sample from line W and 1 sample from line X. *S. aureus* was identified in 1 sample from line Y and 1 from line Z. *S. hominis*was identified in sample from line Y and 1 from line Z. *Enterococcus faecalis* was identified in 2 samples from line X. *S. saprophyticus* was identified in 1 sample from line Y. *Enterococcus hirae* was identified in sample from line Y. *Enterococcus casseliflavus* was identified in 1 sample from line W . *Kurthia gibsonii* was identified in 1 sample from line Y. *Enterococcus faecium* was identified in 1 sample from line Y, and *Staphylococcus* spp. was identified in sample from line Y (Table IV.7).

S. agnetis was identified in 41 samples that were taken from males, and 9 samples that were taken from females. *S. xylosus* was identified in 8 male samples, and 2 female samples. *S. cohnii* was identified in 2. *S. aureus* was identified in 1 male samples. *S. hominis* was identified in 2 male samples. *Enterococcus faecalis* was identified in 1 male and 1 female samples. *S. saprophyticus* was identified in 1 female sample. *Enterococcus hirae* was identified in 1 female

sample. *Enterococcus casseliflavus* was identified in 1 male sample .*Kurthia gibsonii* was identified in 1 male sample. *Enterococcus faecium* was identified in 1 female sample, and *Staphylococcus* spp. was identified in 1 female sample (Table IV.8).

The types of bacteria identified in different bone samples were different based on the type of bone pathological lesion. *S. agnetis* was identified in 17 bone samples with THN, 8 FHN, 7 THNs, 4 FHS, 4 THNc, and in 3 macroscopically normal bone samples. *S. xylosus* was identified in 3 bone samples with FHT, 3 FHS, 3 and 1 normal bone sample. *S. cohnii* was identified in 2. *S. aureus* was identified in 1 male sample. *S. hominis* was identified in 1 sample with FHS, and 1 normal bone sample. *Enterococcus faecalis* was identified in 1 sample with FHN. *S. saprophyticus* was identified in 1 sample with THN. *Enterococcus hirae* was identified in 1 sample with FHN. *Enterococcus casseliflavus* was identified in 1 sample with FHT. *Kurthia gibsonii* was identified in 1 sample with FHS. *Enterococcus faecium* was identified in 1 sample with FHS, and *Staphylococcus* spp. was identified in 1 normal bone sample (Table IV.9).

We examined 40 blood samples, 20 from line Y and 20 from line Z, from apparently healthy birds. Samples were taken at 34 days of age. Thirty four samples were negative for bacterial growth, and 6 samples were positive for bacterial growth. All bacterial positive samples were from males. *S. agnetis* was isolated from 4 samples, 3 from line Y and 1 from line Z. *S. xylosus* was isolated from 2 samples, 1 from line Y and 1 from line Z (Table IV.10). The majority of *Staphylococcus* spp. isolated from lame broilers were closely related on the phylogenetic tree (Fig IV.1).

IV.4 Discussion

In spite of changing the hatchery source for chicks, the results from this experiment generally are in agreement with the results from experiment 1. *S. agnetis* was the predominant bacterial species isolated from all 5 types of tissue samples: right and left femur, right and left tibia, and blood. *S. agnetis* was isolated from 50 (67.6%) samples out of the total 74 analyzed samples from clinically lame broilers. The majority of isolated *Staphylococcus* spp. are closely related on the phylogenetic tree. Homo-infection with *S. agnetis* was the apparent pattern of infection in both males and females. Nine bacterial species out of the total 12 isolated bacterial species were found in Line Y. Line Y is the line with the highest incidence of lameness which might be the predisposition factor for the variety of bacterial species isolated from tissue samples of this line. Further research needs to be conducted to study the reasons for this line's susceptibility to lameness caused by bacterial infection. The majority of lame broilers were diagnosed with FHS and THN. *S. agnetis* was isolated from all of the categories of proximal and tibial head lesions, with the exception of FHT. The fact that we were able to identify *S. agnetis* in broadly different categories of bone lesions indicates that this bacteria is the main, but not the only, cause of lameness in the broilers raised at the University of Arkansas Poultry Research Farm. When examining blood from apparently healthy broilers, *S. agnetis* was isolated from 4 (10%) out of the 40 blood samples. *S. xylosus* was isolated from 2 (5%) out of the total 40 examined samples. The fact that *S. agnetis* and *S. xylosus* are present in the blood of apparently healthy birds constitutes a potential food safety issue which requires more attention from the poultry industry. It is possible that birds exhibiting this level of bacteraemia appeared to be clinically healthy but nevertheless were in the process of developing lameness attributable to BCO. We think that it is important to develop a biomarker to test the blood of healthy birds for

the potential genera and species that can be found in the blood. The use of this biomarker may help avoid food safety issues that might arise from blood-borne bacteria. The presence of bacteria in the blood of healthy birds means that the bacteria has already disseminated to the majority of the tissues. It will be useful to be able to test and eliminate from genetic stocks. We suggest developing a biomarker to be used for genetic selection for resistance to bacterial infection. It was very interesting to find that *S. agnetis* matches are almost all including an unidentified bacterium from *Psoroptes ovis* (a sheep mite) which suggests that sheep might be the other source, in addition to cows, of this species of *S. agnetis*. Also, this finding indicates that *Psoroptes ovis* is one of the vectors that transmit *S. agnetis* or other species of *Staphylococcus* from animals to birds.

Table IV.1. Proximal right and left femoral head lesions in experiment 2. Proximal right and left femoral head (RF & LF) lesions for samples collected from clinically lame broilers in BCO experiment 2. Bone lesion categories include: Femoral Head Separation (FHS), Femoral Head Transitional (FHT), Femoral Head Necrosis (FHN), and Macroscopically Normal.

Type of infection	RF	%	LF	%
FHS	11	40.7	8	29.6
FHT	5	18.5	5	18.5
FHN	4	14.8	11	40.7
Macroscopically Normal	7	25.9	3	11.1
Total	27	100.0	27	100.0

Table IV.2 Proximal right and left tibial head lesions in experiment 2. Proximal right and left tibial (RT & LT) head lesions for samples collected from clinically lame broilers in BCO experiment 2. Bone lesion categories include: Tibial Head Necrosis (THN), severe Tibial Head Necrosis (THNs), caseous Tibial Head Necrosis (THNc), and Macroscopically Normal.

Type of infection	RT	%	LT	%
THN	17	63.0	12	44.4
THNs	2	7.4	8	29.6
THNc	3	11.1	3	11.1
Macroscopically Normal	5	18.5	4	14.8
Total	27	100.0	27	100.0

Table IV.3 Total number of successfully analyzed tissue samples (out of 27 collected per category) in BCO experiment 2, and percentage of samples analyzed by tissue type. Tissue samples include: Blood, Right Femur (RF), Left Femur (LF), Right Tibia (RT), and Left Tibia (LT).

Type of tissue sample	Total number of analyzed samples	%
Blood	8	10.8
RF	15	20.3
LF	14	18.9
RT	15	20.3
LT	22	29.7
Total	74	100.0

Table IV.4 Type of infection based on bird's gender in BCO experiment 2

Infection	Male	%	Female	%
Homogenous	9	47.4	5	62.5
Mixed Species	7	36.8	0	0.0
Mixed Genera	3	15.8	3	37.5
Total	19	100.0	8	100.0

Table IV.5 Types of bacterial species identified in tissue samples in BCO experiment 2

Species	Total number	%
Enterococcus faecalis	2	2.7
Staphylococcus agnetis	50	67.6
Staphylococcus aureus	2	2.7
Staphylococcus hominis	2	2.7
Staphylococcus saprophyticus	1	1.4
Staphylococcus xylosus	10	13.5
Enterococcus hirae	1	1.4
Enterococcus casseliflavus	1	1.4
Kurthia gibsonii	1	1.4
***Staphylococcus* spp.**	1	1.4
Enterococcus faecium	1	1.4
Staphylococcus cohnii	2	2.7
Total	74	100.0

Table IV.6 Types of bacterial species identified based on tissue in BCO experiment 2. Tissue samples include: Blood, Right Femur (RF), Left Femur (LF), Right Tibia (RT), and Left Tibia (LT).

Bacteria	RF	LF	RT	LT	BL	Total
Enterococcus faecalis	1	0	0	0	1	2
Staphylococcus agnetis	5	8	14	16	7	50
Staphylococcus aureus	1	0	0	1	0	2
Staphylococcus hominis	1	1	0	0	0	2
Staphylococcus saprophyticus	0	0	0	1	0	1
Staphylococcus xylosus	3	3	1	3	0	10
Enterococcus hirae	1	0	0	0	0	1
Enterococcus casseliflavus	0	1	0	0	0	1
Kurthia gibsonii	1	0	0	0	0	1
Staphylococcus spp	0	0	0	1	0	1
Enterococcus faecium	1	0	0	0	0	1
Staphylococcus cohnii	1	1	0	0	0	2
Total	15	14	15	22	8	74

Table IV.7 Bacterial distribution based on broiler line in BCO experiment 2. Line distribution for examined birds was 8 birds from line Y, 7 birds from line Z, 7 birds from line W and 5 birds from line X.

Bacteria	Y	Z	W	X	Total
Enterococcus faecalis	0	0	0	2	2
Staphylococcus agnetis	8	13	18	11	50
Staphylococcus aureus	1	0	1	0	2
Staphylococcus hominis	1	1	0	0	2
Staphylococcus saprophyticus	1	0	0	0	1
Staphylococcus xylosus	5	3	0	2	10
Enterococcus hirae	1	0	0	0	1
Enterococcus casseliflavus	0	0	1	0	1
Kurthia gibsonii	1	0	0	0	1
Staphylococcus spp	1	0	0	0	1
Enterococcus faecium	1	0	0	0	1
Staphylococcus cohnii	0	0	1	1	2
Total	20	17	21	16	74

Table IV.8 Bacterial distribution based on broiler gender in BCO experiment 2

Bacteria	Male	%	Female	%	Total	%
Enterococcus faecalis	1	1.8	1	5.9	2	2.7
Staphylococcus agnetis	41	71.9	9	52.9	50	67.6
Staphylococcus aureus	1	1.8	1	5.9	2	2.7
Staphylococcus hominis	2	3.5	0	0.0	2	2.7
Staphylococcus saprophyticus	0	0.0	1	5.9	1	1.4
Staphylococcus xylosus	8	14.0	2	11.8	10	13.5
Enterococcus hirae	0	0.0	1	5.9	1	1.4
Enterococcus casseliflavus	1	1.8	0	0.0	1	1.4
Kurthia gibsonii	1	1.8	0	0.0	1	1.4
Staphylococcus spp	0	0.0	1	5.9	1	1.4
Enterococcus faecium	0	0.0	1	5.9	1	1.4
Staphylococcus cohnii	2	3.5	0	0.0	2	2.7
Total	57	100.0	17	100.0	74	100.0

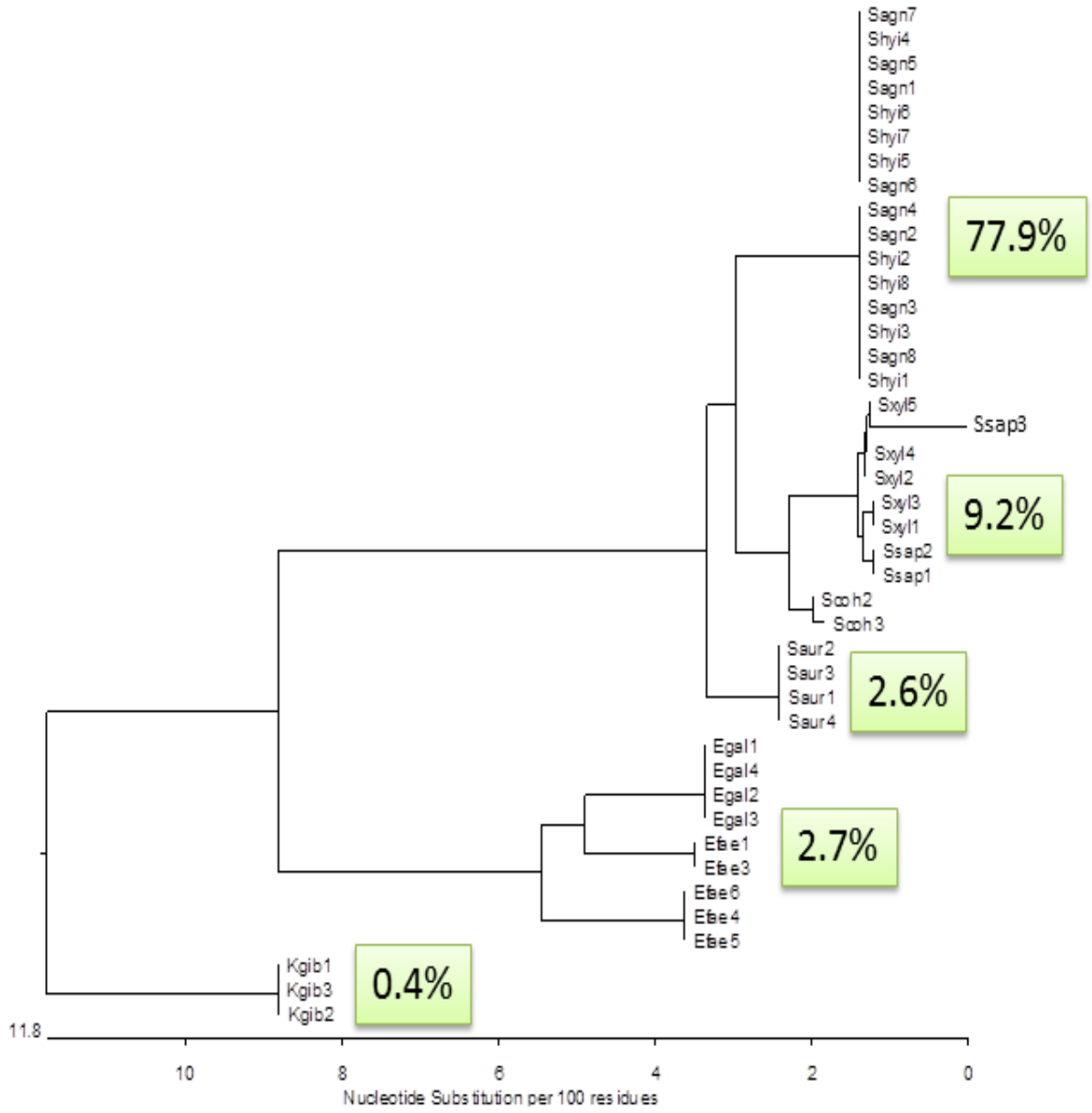
Table IV.9 Bacterial distribution based on bone lesion in BCO experiment 2. Bone lesion categories include: Femoral Head Transitional (FHT), Femoral Head Necrosis (F HN),Femoral Head Separation (FHS), Tibial Head Necrosis (THN), severe Tibial Head Necrosis (THNs), caseous Tibial Head Necrosis (THNc), and Macroscopically Normal(Nor) .

Bacteria	FHT	FHN	FHS	THN	THNs	THNc	Nor	Total
Enterococcus faecalis	0	1	0	0	0	0	0	1
Staphylococcus agnetis	0	8	4	17	7	4	3	43
Staphylococcus aureus	0	0	1	0	0	0	1	2
Staphylococcus hominis	0	0	2	0	0	0	0	2
Staphylococcus saprophyticus	0	0	0	1	0	0	0	1
Staphylococcus xylosus	3	0	3	3	0	0	1	10
Enterococcus hirae	0	1	0	0	0	0	0	1
Enterococcus casseliflavus	1	0	0	0	0	0	0	1
Kurthia gibsonii	0	0	1	0	0	0	0	1
Staphylococcus spp	0	0	0	0	0	0	1	1
Enterococcus faecium	0	0	1	0	0	0	0	1
Staphylococcus cohnii	0	0	1	0	0	0	1	2
Total	4	10	13	21	7	4	7	66

Table IV.10 Bacterial species isolated from blood of apparently healthy birds in BCO experiment 2

Bacteria	Y	Z	Total
Staphylococcus agnetis	3	1	4
Staphylococcus xylosus	1	1	2
Total	4	2	6

Fig IV.1: Phylogenetic tree of isolated bacterial species



V. References

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