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*University of Arkansas, Fayetteville*

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Bacterial Chondronecrosis with Osteomyelitis in broilers: genomics, phylogenomics, and  
methods to detect specific pathogens during outbreaks.

A dissertation submitted in partial fulfilment  
of the requirements for the degree of  
Doctor of Philosophy in Cell and Molecular Biology

by

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## ABSTRACT.

Lameness is a major issue in animal welfare and the broiler industry. Bacterial chondronecrosis with osteomyelitis (BCO) is one of the main causes of lameness. Many staphylococcal species, including *Staphylococcus agnetis* isolate 908, have been isolated from the bones and blood of lame broilers at the University of Arkansas. *Staphylococcus agnetis* is a coagulase-variable, Gram-positive bacterial species that has been previously associated with subclinical or mild clinical cases of mastitis in dairy cattle. The annotated complete genome of hypervirulent strain 908 was published at NCBI. In this study, it has been compared to nine genomes we assembled for hypervirulent isolates in dairy cattle. Phylogenomic analyses of chicken and cattle isolates of *S. agnetis* and *Staphylococcus hyicus* suggest a very close relationship between the cattle and chicken isolates. The hypervirulent chicken isolate, 908, clustered with two of the cattle isolates, including strain 1379. A catalogue of gene differences between the cattle and chicken isolates was constructed using reciprocal blast analyses at the nucleotide and polypeptide level. More than 40 genes and 3 plasmids from strain 908 are absent or poorly conserved in any of the cattle *S. agnetis* isolates. No transformation protocol has been described for *S. agnetis*. Subsequently, an electroporation procedure has been optimized for DNA transformation of *Staphylococcus agnetis*. Therefore, we have optimized an electroporation method for DNA transformation so that we regularly obtain 10 to 20 transformants per ng using a Gram+/Gram- shuttle vector. Moreover, among the BCO pathogens isolated from the lame broilers, there are a number of *Staphylococcus* species, such as, *S. agnetis*, *S. hyicus*, *S. chromogenes*, *S. aureus*, *S. cohnii*, *S. saprophyticus*, *S. epidermidis*, and *S. capitis*, which are hard to accurately identify based just on genes like 16S rDNA. Therefore, using *pfbA* gene, a novel PCR assay was optimized for *Staphylococcus* species discrimination and strain typing. Moreover, extraction of bacterial DNA for subsequent molecular diagnostic applications remains

a costly and time-consuming operation. We developed a technique for rapidly extracting genomic DNA from the BCO pathogens and other environmental bacteria based on sodium hydroxide cell lysis with or without magnetic bead capture. Finally, the BCO pathogens are transmitted via air. Our efficient air sampling system was designed for the quick screening of these airborne BCO pathogens and is transferable to monitor agriculturally important pathogenic bacteria.

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## **DEDICATION.**

I dedicate this dissertation to my lovely wife Bafreen Shwan, with whom I have shared the burden of my life, and it is an honor to share my love, life, and business with her; to my kids, Laveen, Ahmed, and Aland who will inherit this world and make it a much better place, their growth provides me with an endless source of pride and happiness.



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## LIST OF PUBLISHED ARTICLES

### **Chapter 2.**

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## **CHAPTER 1**

### **Introduction**

### **Literature Review**

## **Chapter 1: Introduction. Literature review.**

In normal life, prior to the COVID-19 pandemic, human malnutrition continues as a significant public health issue in the world, causing more than 3 million avoidable maternal and infant deaths annually (de Bruyn et al., 2015). Also, the consumption of chicken meat has also been growing year after year (Tavárez & Santos, 2016). According to the UN report of 2019, the world population will increase from 7.4 billion in 2017 to 9.7 billion in 2050. This rapid rise in the world population will increase the demand for food (United Nations 2019; Fukase & Martin, 2020; Daszkiewicz et al., 2022). Side by side with the increased human population, the poultry industry is also growing around the globe. In 2019 and 2020, it reached 131.6 and 133.3 million tons, respectively (FAO, 2020). However, the increase in global hunger and malnutrition in 2020 was mostly caused by COVID-19. In the last two years, the sustainability of poultry production has been severely influenced by the COVID-19 pandemic, especially in developing countries (Attia et al., 2022).

The COVID-19 pandemic had a significant impact on poultry production industries, the supply of food, and the demand for it in a number of countries, including the United States, the European Union, India, China, Spain, the United Kingdom, Italy, and Germany. COVID-19 has ruined the poultry and dairy industries in Canada (Fig. 1.1; Attia et al., 2022; FAO, 2019; Weersink et al., 2021).

Poultry meat and eggs produce high-quality protein, significantly improving the nutritional adequacy of conventional staple-crop diets. However, the worldwide issues of food security, population growth, food quality and safety, environmental concerns, and limited resources should be addressed (Wang et al., 2017; de Bruyn et al., 2015). To avoid a catastrophe by 2030, especially in Africa, the world must take action now (WHO, 2021). The long-term



sustainability of poultry production while preparing for unexpected circumstances such as COVID-19 is a critical need for the sector.

Poultry are birds that humans domesticate for their meat, eggs, and feathers (Vaarst et al., 2015), which comprise chicken, fowl, turkey, guinea, peafowl, duck, goose, pigeon, pheasant, and quail (Miao et al., 2013). Chickens are the most common species of poultry that makeup 90% of the poultry population worldwide (Alders et al., 2018). Chicken is one of the most stable and widely used sources of animal protein (eggs and meat) in the world today (de Bruyn et al., 2015; Alnahhas et al., 2016; Marangoni et al., 2015).

### **Modern chickens ancestry.**

Some evidence is available that indicates a number of chicken domestications were started in Southeast Asia, South Asia, and southern China from the end of the Pleistocene to the beginning of the Holocene era (Miao et al., 2013; Tixier-Boichard, et al, 2011; Bosse, 2019; Peters et al., 2016). *Gallus gallus domesticus* (modern chicken) is the most common type of poultry that is domesticated worldwide. The mitochondrial DNA evidence collected in the early twenty-first century suggests that different red junglefowl (*Gallus gallus*) subspecies are the wild forebears of the modern chicken. However, based on more recent genetic evidence, *Gallus gallus spadiceous* is the main wild ancestor. This species is found in northern Thailand, southern China, and Myanmar (Eda, 2021).

Archaeological data and the sequence-based phylogenetic tree constructed of the mitochondrial DNA (mtDNA) of modern chickens implies that the domestic chickens first originated in Southeast Asia more than 8000 years ago, and they were eventually spread around

the globe by sailors and merchants through land and sea from 1400-900 BC. They are now the world's most significant poultry species (FAO, 2020). The bones of domestic chickens have been discovered at archaeological sites dating back to the early and middle Holocene periods. However, their legitimacy remains disputed. Also, direct radiocarbon dating and species identification are needed to authenticate their reality (Eda, 2021). In 2017, the world's chicken population was more than 22 billion birds. Asia was home to around 56 % of the total number of birds (FAO, 2020). Chickens accounted for around 93 % of the world's poultry population in 2019, with ducks (4 %) and turkeys (2 %) following closely behind (FAO, 2020).

The chicken was domesticated from a subspecies of jungle fowl found in Southeast Asia, the red jungle fowl, the primary parents of the most recent domestic chickens and the most widely spread poultry species, began as early as 5400 BC (7, 000-10, 000 years ago) in Southern China or Southeast Asia, where it was an important part of the development of human culture. The zoological and archaeological evidence also proves that chickens were domesticated in Africa, especially in ancient Egypt between 1307–1196 BC (Mwacharo et al., 2013; Houlihan & Goodman, 1986).

The earliest domesticated red junglefowls may have happened in the region where the species occurs, particularly in the area where *G. g. spadiceus* was spread. Because it's possible that red junglefowl lived through different stages of domestication, it is believed that bone analysis will help us learn more about their history and interactions with humans in the area (Eda, 2021). According to some hypotheses, the modern chicken developed from a polyphyletic ancestor that included two or more of the wild junglefowl species that may still be found today in their natural habitat (Hutt, 1949).

A single-species origin for domestic chicken has been shown via molecular analysis among modern domestic chicken, red and gray junglefowls (Baker, 1968). The red junglefowl and domestic chickens have a tight genetic link, according to molecular investigations, since they produce egg proteins that are quite similar to each other. On the other hand, the gray junglefowl and domestic chickens were quite different in their G2 globulin. As a result, the red junglefowl is the main ancestor of the domestic fowl (Baker, 1968). An extensive phylogenetic examination of domestic chickens and four *Gallus* junglefowls, done using blood protein and DNA fingerprinting methods, revealed a close relationship between the modern chicken and the red junglefowl. An examination of the 400 base pair of mtDNA sequence in four wild junglefowl species and nine domestic chicken breeds revealed a monophyletic relationship between domestic chicken and red junglefowl, which was previously thought to be a result of hybridization (Sawai et al., 2010; Fumihito et al., 1996).

This makes chickens an important biomarker of societies' and civilizations' trade, cultural relations, and agriculture. Archaeological evidence shows that chickens also came to Europe from southern (Greece and Persia) and northern (China and Russia) trade routes (West & Zhou, 1988; Zhang, et al, 2017; Crawford, 1990; Muchadeyi et al., 2008; Fumihito et al., 1994; Xiang et al., 2014; Storey et al., 2010).

The spread of chickens in America are disputable (Miao et al., 2013). Some reports have shown that evidence of DNA and carbon dating indicates that from pre-Columbian AD 1304-1424, Polynesian chickens were introduced into South America (Chile) (Storey et al., 2007). Storey et al. (2007) argued that chickens were transported to the Americas prior to the advent of the Spanish or Portuguese. Gongora et al., (2008) also refuted the view of a Polynesian-Chilean

American connection, citing that sequences of pre-Columbian chickens lie between European/Indian subcontinental and Chinese haplotypes rather than Polynesia.

### **Chickens domestication.**

The domestication of wild animals marks a significant turning point in human history (Hata et al., 2021; Diamond, 2002). The modern chicken (*Gallus gallus domesticus*) is the most widely domesticated animal on the planet, yet it is unclear when and where it was initially domesticated by humans (Eda, 2021). Broilers, which are grown particularly for meat and have a high feed-to-meat conversion ratio, are the birds that are most often produced by the contemporary integrated poultry industry (FAO, 2020).

Over time, the domestication of chickens has become common in the South and Southeast Asian regions (Granevitze et al., 2009). It is debatable where and when chickens as a major species of poultry were first used for economic purposes. Chicken is raised for its eggs and meat (Fumihito et al., 1994; Rubin et al., 2010). The domesticated chicken was originally performed for various uses, such as for rituals, the use of a crowing large rooster to signal the morning hour, food (meat and eggs), symbolic, cultural and religious, decorative purposes, and entertainment (cockfighting), religious, and there is a great deal of variation in morphological traits across breeds in the Middle East, Southeast Asia, and Africa. Also, in Europe and USA, chickens have been used by poultry breeders in their exhibitions. Therefore, chickens were chosen for unique characteristics, such as coloured feather designs and uniformity. A variety of cockfighting and pet breeds have been developed and bred all across the globe (Crawford, 1984; Elferink et al., 2012; Núñez-León et al., 2019).

These attempts have greatly promoted the improvement of standard chicken breeds and chicken varieties that are used in today's chicken production, which is the most diverse of all domestic species. The selection of chickens with key features in terms of meat and egg production began in the early 1900s. Also, to study human diseases, chickens have been utilized as good biological and medical models, especially in Europe and the USA (Perry-Gal et al., 2015; Serjeantson, 2009).

Before World War II, the production of chicken meat was a secondary market for the egg industry. Both the increased availability of feed materials and market demand for chicken meat during the period of red meat restrictions contributed to the rudimentary poultry industry's early development. As the largest producer of chicken meat, the U.S. poultry industry is one of the country's most successful agricultural sectors (National Chicken Council, 2022). In countries, such as India, Korea, China, Thailand, and Japan, chicken domestication has increasingly become very popular (Liu et al., 2006; West & Zhou, 1988; Kanginakudru et al., 2008).

### **Broiler industry.**

In the 1800s, as a part of backyard farming in the U.S., chickens were mainly raised in small flocks in households. The chickens were used for both meat and egg, production. The large-scale commercialization of the poultry industry began in the 1950s. Prior to that, the chicken had been a dual-purpose animal with meat production and reproductive characteristics that were complimentary to one another. However, the realization that these two characteristics are not complementary contributed to the establishment of the new poultry business that distinguishes the egg-type and meat-type birds. One of the most successful sectors in U.S. agriculture production in the United States of America is the poultry industry (Griffin & Goddard, 1994).

The commercialization of poultry started in the early twentieth century with a broiler house that accommodated up to 10, 000 birds. Poultry Processing in the U.S., such as most other parts of the world, was mostly performed in small, non-specialized units using different chicken breeds that already existed on the continent at the beginning of the 1900s. From the late 1930s to the mid 1940s, throughout Europe and in the US, there was a surge in poultry production (Sansbury, 2000). Since the mid-1900s, when commercially accessible items began to be scrutinized, the use of chicken meat has steadily grown in popularity. However, it was not until the 1970s that major developments in management methods, automation technology, breeding programs, genetic selection, nutritional discoveries, and disease eradication programs enabled the industry of commercial poultry to produce poultry products of high quality at reasonable prices (Chambers et al., 1981).

In the mid-1990s, per capita intake of chicken meat exceeded that of beef and pork in terms of pounds per year (MacDonald, 2008). In the early twentieth century, animal genetics and breeding procedures were mostly applied for commercial purposes in the livestock processing industry (Johnson & Ruttan, 1997). In the last 50 years, the poultry industry has continued to grow, with per capita chicken consumption in the U.S. rising from 30 to 110 pounds (Fig. 1.2; USDA, 2022). However, beef consumption has declined marginally during the same period, while pork and turkey consumption has stayed constant (Meyer & Steiner, 2011). Broilers, turkeys, chickens, and eggs added \$38.7 billion to global revenue in 2016 (U.S. Poultry and Egg Association, 2016). Due to developments in quantitative genetics and the popularity of the poultry industry's commercial objective, the largest development in chicken genetics for the large-scale commercialization of the poultry industry was witnessed in the second half of the 20th century (Tallentire et al., 2016).

Part of the reason for the change in eating habits in the U.S. is the price difference. In December 2015, the average price of beef per pound was \$5.50, while the average price of chicken per pound was \$1.94. (Hahn, 2016). Compared to beef and pork, chicken meat is more affordable on a global scale. Chicken meat was 32% and 48% cheaper than beef (\$5.89/lb) and pork (\$3.88/lb), respectively, according to USDA Economic and Research Service (2019). In addition, other related factors, such as the increase in public health consciousness (a healthy alternative to red meat), the lack of cultural/religious limitations on chicken meat intake, the growing scale of the human population, and the effectiveness of production all play a part (Tallentire et al., 2018). Massive advances have been made in economically important characteristics, such as breast file yield. Feed conversion decreased from 4.42 in a 1957 broiler population to 1.47 in 2003. and the time it takes for a broiler to hit 1.5 kg of live weight decreased from 120 days to 30 days in 1925 versus 2005 (Albers, 1998; Havenstein et al., 2003). As a result, more eggs have been produced by layers and the number of raising days has been cut down, which has led to better production profitability. This has allowed the birds to reach market weight earlier than ever before ( Tallentire et al., 2018; Buzala & Janicki, 2016).

Prevention of disease and appropriate animal husbandry have both had a role in decreasing mortality rates over the years (Flock & Preisinger, 2007). Both of these developments have had a negative impact on the poultry industry for the last 30 years. Divergent growth rates cause ascites syndrome (Julian, 2005), fat deposition (Barbut, 1997), bone deformation (Julian, 1998), reproductive inefficiency (Emmerson, 1997), and immune system defects (Leshchinsky & Klasing, 2001), to name a few consequences that lead to economic losses in the poultry industry.

The rapid growth rate in the U.S. broiler industry, which processed over 36 billion pounds of broiler meat in 2007, contributed to the sector's competitiveness (Schmidt et al.,

2009). The demand for poultry products around the world is continuing to rise at an astounding pace. This is due to the food's high nutritional value and low cost. Over time, there has been a substantial change in body weight. It was found that a 42-day-old fowl from 1950 was 539 grams in weight, whereas a typical modern broiler of the same age is around 3000 grams (Havenstein et al., 2003). Broilers are the most common type of poultry in the world (Tavárez & Santos, 2016). It accounts for 70% of the market, whereas turkey and other poultry account for approximately 8% and 22%, respectively. This illustrates that broiler meat is superior to other poultry meats.

This degree of global integration facilitates international trade, especially through common policies and technologies, to ensure continuity in poultry practices and products (Bessei, 2018). In most cases, integrated production requires contracting production to local breeders. In the year 2000, the world's poultry production produced 57 million tons of chicken meat. Approximately 128 million tons of chicken meat were produced around the world in 2019 (FAO; Executive Guide to World Poultry Trends, 2022). In 1968, the poultry industry in the U.S. produced about 9 billion pounds (4.5 million tons) of chicken meat, but in 2018, it produced 56 billion pounds (28 million tons), representing a 600% increase in productivity over the previous year (Fig. 1.1; USDA, 2019). Poultry production in the U.S. was estimated at \$32 billion in 2014 and still continues to rise (Fig. 1.3).

The supply chain for a breeder entails anything from primary breeders to the industry's genetic stock. The elite (pedigree/foundation), great grandparents (GGP), and grandparent (GP) generations of these breeders are owned by major broiler breeding corporations, such as Cobb-Vantress and Aviagen. Eggs from pedigree flocks are hatched in a specific pedigree hatchery, and the progeny goes on to grow into GGP flocks (USDA, 2013). Eggs from the GGP line are utilized to produce chicks of the GP generation. Grandparent flocks are two-way cross hybrids



with male and female lines from two separate lines. Day-old chicks from GP stock are sent to specialized GP hatcheries across the country to create the last generation of breeding population (PS; multiplier/parents/broiler breeder), which is then shipped to the agricultural field, where the eggs are hatched to create birds for human consumption (broilers, layers, and market turkeys; Fig. 1.4).

Primary breeding stocks are kept on high-level biosecurity farms because of their relevance and critical position in the growth pyramid (Fig. 1.6; Paxton et al., 2010). According to the European Food Safety Authority (EFSA), breeding enterprises may impact the health and robustness of broiler chickens as well as their competitiveness via genetic selection. In the great majority of commercial broiler breeding systems, a four-way cross is used (Jiang et al., 1999). The use of systematic matings, including the crossing of various breeds, strains, or inbred lines, is carried out in order to demonstrate hybrid vigor or heterosis exploitation. Sire lines, for example, are chosen because they can grow. Dam lines, on the other hand, are chosen because they can produce eggs (Jiang et al., 1999). The result of crossbreeding is a large number of healthy chicks that suit the needs of poultry farmers and producers. Each dam will produce more than 200 chicks every generation, resulting in a greater rate of genetic development in broilers (USDA, 2013).

Broiler production revenues have risen in recent years, to the point that broiler farmers now earn more than non-farm workers. Poultry production has a sizable market, resulting in significant increases in farmers' expenditures per square foot of farm as a result of the increased daily weight growth performance of the birds. Also, there was no obvious effect on the quantity of feed needed per bird. Despite a turnover of only 6% of the broiler breeders, there is a long waiting list of farming households looking for jobs in the poultry sector (USDA, 2022).

In 2022, the number of eggs put into hatcheries was higher compared to the same time in 2021 in the US. A total of 241 million eggs were placed in incubators by hatcheries in the United States for the week ending March 5, 2022. The percentage of hatched eggs was 79.3%. The average was found by dividing the number of chicks hatched by the number of eggs placed in hatcheries over the last three weeks prior to the hatch (USDA, 2022).

### **Schemes of modern broiler breeding.**

Current chicken breeds are the result of billions of years of natural selection, with the advent of genetic selection of suitable characteristics for commercial purposes (Tallentire et al., 2016; Schmidt et al., 2009). The commercial broiler selection process has improved (Paxton et al., 2010). Since the beginning of the twentieth century, better management, nutrition evaluation, and breeding programs have been the foundation for progress in the chicken production business. This trend has continued into the present day (Hutt, 1949; Bessei, 2006; Titus, 1941). Multiple selection requirements for growth-related traits that favorably affect economic gains and match the demands of the customer were included in the selection program in the USA, rather than relying exclusively on basic mass selection for live weight at market age.

Selective breeding of chickens using progeny testing to improve quantitative traits began in the 1940s because there was more demand for chicken meat (Hunton, 2006). Commercial genetic firms emerged, and they started focusing on selecting characteristics related to disease tolerance, egg production, feed effectiveness, meat yield, and meat quality, among other traits (Albers, 1998).

There have been a variety of genetic enhancement protocols launched. Poultry breeders have used line- and cross-breeding strategies taken from the hybridization techniques of plant breeders (Sansbury, 2000). The number of poultry breeding programs declined after the successful implementation of crossbreeds. Poultry breeders have become streamlined and dedicated to raising large-scale poultry (Sainsbury, 2000).

The two main technologies that have contributed to the fruitful raising of poultry are trap-nest and artificial incubators. In addition to quantitative genetics, modern broiler breeding firms have created a breeding and distribution structure for broilers that can be visualized as a pyramid (Fig. 1.5). Since the 1950s, pyramid breeding has led to the production of lines that have experienced a 300% increase in growth rate (Paxton et al., 2010). Each stage of the pyramid reflects one generation (Fig. 1.5), and from one male and ten females, approximately 50 million broilers are generated at the fifth generation (Laughlin, 2007; Flock & Preisinger, 2007). Over 50 efficiency, production, and general health traits are assessed for each pedigreed offspring in the Cobb-Vantress Inc. (Siloam Springs, AR) breeding program (Katanbaf & Hardiman, 2010).

Since economically significant characteristics had strong heritabilities, the initial focus on mass selection culminated in a positive response. This genetic effect resulted in rapid positive responses in pedigreed selection systems in commercial broilers (Hunton, 2006). However, the introduction of industrial farming at the turn of the century helped in choosing high-yield meat breeds (Schmidt et al., 2009). When compared to their wild ancestor, the Red Jungle fowl, chicken body mass and growth traits have increased from twofold to more than fivefold during domestication, particularly in the latter stages of breed improvement in the U.S. (Johnsson et al., 2018).

It was found early on in the history of what is now known as the predecessor of the modern-day poultry industry that growth-related traits and reproduction-related traits have a negative association (Siegel et al., 2006). Consequently, breeds that were initially selected as dual-purpose breeds (with a focus on meat yield and egg production) have been replaced by breeds that have a specialized selection. Since then, top broiler breeding companies have employed quantitative genetics to increase growth rate, meat yield, and feed conversion in their flocks. As a result, the Cornish Cross (chickens need special care and nutrition, yet, they grow at a remarkable rate) now accounts for the bulk of the broiler market in North America, and its range has resulted in a bird that reached market weight at day 42. With the growth of the commercial poultry industry and increasing market demand for low-cost poultry, new breeding systems emerged in which chickens were genetically selected for suitable production traits for a particular reason. Broiler chicken lines, for example, were designed primarily for meat production, while layer hens were developed specifically for egg production. Every year, genetic selection has lowered the age at which broilers reach slaughter weight by up to one day. Figure 1.6 shows that the growth rate of broiler chickens grew by almost 400 % between 1950 and 2005, whereas the feed conversion ratio (FCR) declined by 50 % (Zuidhof et al., 2014; Buzala & Janicki, 2016). The genetic selection also increases pectoralis minor muscle yield by 30% in males and 37% in females. It also increases pectoralis major muscle yield by 79% in males and 85% in females. The pedigree range of genetically enhanced chicken lines is determined by the reason for which the flocks are raised. The number of eggs per day, egg type, egg weight, eggshell width, yolk colour, index, Haugh unit, and albumen index are all traits that are commercially advantageous for business. Genetic techniques are used to pick chickens that have

these traits, as well as other important traits, such as the hens' ages of sexual maturity and clutch time (Suma et al., 2007; Lukanov, 2014; Bahmanimehr, 2012).

Modern commercial broilers are highly specialized lines produced by crossing pedigree (pure) lines over several generations to acquire a broad variety of beneficial meat-producing traits (Deeb & Lamont, 2002; Paxton et al., 2010). Such long-term and scientific breeding schemes have increasingly been made possible by new technologies and facilities, as well as major developments in population genetics (Havenstein et al., 2003).

Today's broiler goods are usually three- or four-generation crosses between pure breeding lines (Paxton et al., 2010).

The typical generation and multiplication pyramid from pure breeding line to broiler products includes pure-breeding lines (great-great grandparent lines), great-grand parent stock, grandparent stock, parent stock, and broilers. For each stage, a continuous gene flow occurs, and the next level is created as a product of a cross of the genetically most compatible males and females at that level. Chickens owned by major breeding businesses are kept on biosecure farms for the purpose of selection systems. For their multiple broiler lines, breeding companies retain up to ten pure-breeding lines. Pure lines of genetically selected desired characteristics are at the top of the development pyramid. Male and female lines are normally chosen for their unique characteristics at each division of the pyramid. Great-grand parent stock is derived from pure-breeding lines and is mainly used to multiply the line and create tens of thousands of offspring, which are needed to generate grandparent lines. For such characteristics, they are subjected to mass selection. The key breeding firms have a tight grip on them. Both male and female broiler lines are developed for features that are somewhat different from one another; males are mostly selected for heritable growth and feed efficiency traits, while females are primarily selected for

reproductive success traits (Olori, 2009). They have labeled the flocks A females and males, B females and males, C females and males, and D females and males in Figure 1.4. In the four-way ABCD cross, grandparent stock birds are the first generation. They are A males x B females and C males x D females from great-grandparent stocks used to make AB or CD hybrid parents. Hundreds of thousands of grandparent stocks are sold to parent stock distributors or organized manufacturing firms in the local area. The parent stock consists of AB-hybrid males and CD-hybrid females. Broiler manufacturing firms purchase and operate the majority of them. On the other hand, broilers are the commercial products of cross-breeding parent stocks. They are broilers that have been bred, slaughtered, and processed in vast quantities for human consumption Fig. 1.5; Muir & Aggrey, 2003).

Poultry breeding targets are often guided by expectations for potential meat production. As a result, genetic selection is becoming more prevalent in modern broiler processing (Paxton et al., 2010). Poultry breeders held up with the rate of demand throughout the twentieth century by adapting to a variety of critical selections and breeding technical advances. It is projected that 400,000 pedigreed individuals representing 35–40 purebred lines from different firms will be the progenitors of approximately 400 billion commercial broilers on a global scale (Pollock, 1999). Developing a stable, better commercial broiler line will take 4.5 years if it is based on pure lines with varying reproductive lifespans. After more than 60 years of genetic selection, modern breeding techniques have produced exceptionally appealing, resistant lines that have increased in size by more than 300% while maintaining the proper body shape (Paxton et al., 2010).

To recap, today's industrial broiler is the outcome of production schemes and massive improvements in management. Artificial genetic selection approaches targeted at developing

genetically modified chicken lines with improved quantitative development as well as highly heritable qualitative features have gained favour over the last several years.

### **The pros and cons of genetic selection in broiler production.**

The poultry industry has had a lot of growth, due to its ability to produce suitable products at a reasonable cost (Anthony, 1998). This performance is the result of a number of factors, such as more intense selection, shorter generation cycles, and less environmental consequences (Tallentire et al., 2018). When breeding broilers, it is important to look at both the benefits and the drawbacks that have come from very strict breeding.

The poultry meat industry's performance in producing high-quality, inexpensive food in large amounts following WWII is well known (Hocking, 2014). Commercial selection has been extensive in the modern poultry sector, resulting in improved quality and performance. This has led to a big rise in the growth rate or volume of weight gain over time and a noticeable change in body shape over the last few decades (Paxton et al., 2010).

The average number of eggs laid by commercial layers is approximately 300 per year, but indigenous chickens typically produce about 40–60 eggs per year (FAO, 2020). Approximately 1600 distinct indigenous chicken breeds exist across the globe. Also, there are about 270 different duck breeds, 200 different goose breeds, and 110 different turkey breeds. They are the consequence of hundreds of years of natural selection, cross-breeding, and in-flock breeding among flocks (FAO, 2020).

Since the early twentieth century, the time required to create a new market-weight broiler has fallen considerably. In the 1920s, raising 2.5-pound live-weight birds took an average of 112

days. In 2019, six-pound live-weight chicks will be grown in 47 days (Muir & Aggrey, 2003). In 2019, the "Feed to Meat Benefit," or the amount of feed required to produce a pound of live weight broiler, was around 4.7 pounds of feed per pound of broiler (NCC, 2020). The advantage of feeding meat was 1.80 lb per pound of broiler production in 2019, giving a respectable meat yield (NCC, 2020). Since the 1950s, contemporary broilers have been bred for their rapid growth rate. By 2015, the rate of development had accelerated by 400%. (Renema et al., 2007; Fig. 1.2). Along with meat quality and quantity, the monetary value and exports of broilers have been significant (USDA, 2019). Since 1925, chicken mortality has fallen from 18% to 5%. (NCC, 2020). Surprisingly, broiler meat production has had a lower environmental impact than beef and pig processing (Anthony, 1998), owing to lower greenhouse gas emissions from fossil fuels used in feed processing and less nitrogen depletion from chicken manure. These benefits are magnified for feed-efficient birds (Tallentire et al., 2018).

Many studies have recorded unfavourable correlated responses to intensive poultry production systems linked to genetic selection, such as ascites or pulmonary hypertension syndrome (PHS) (Wideman & French, 1999), sudden death syndrome (Schmidt et al., 2009; Hocking, 1993), and skeletal deformities (Zuidhof et al., 2014). Ascites is a condition aggravated by increased pulmonary pressure and associated hypoxia that leads to a fluid concentration in the peritoneal cavity, resulting in abdominal swelling (Al-Zahrani et al., 2019; Wideman, 2000). Muscle anomalies resulting from the development of high-yield birds heavy with meat that exceeds many metabolic and /or anatomical limits are another concern arising from and correlated with broiler rapid growth rates (Anthony, 1998). These studies show that human-directed evolution, such as genetic selection for higher development capacity, can disrupt genetic homeostasis, resulting in decreased health and increased mortality, as well as low walking



activity or locomotion (Tickle et al., 2014). Additionally, these tests demonstrated how selection for fast development and early carcass formation impacted individual tissues and organs, and increased our knowledge of how alterations in one organ system may have pleiotropic consequences on others (Schmidt et al., 2009). More data is needed to help explain how accelerated growth and body mass have affected the anatomical and physiological improvements in broilers as they progress through ontogeny (Tickle et al., 2014).

In terms of animal behaviour, little disparity was reported in resting temperament between fast-growing and slow-growing broilers raised to 13 weeks, since the birds were motivated to engage in a variety of behaviors in a safe atmosphere. Broilers of all ages were unable to do as many of these tasks as they aged, most likely due to their weight (Bokkers & Koene, 2003). Over the past three decades, the occurrence of breast meat abnormalities such as wooden breast (WB), white striping, and spaghetti meat (SM) has increased in broilers (Abasht et al., 2016; Sihvo et al., 2017). Sihvo et al. classified woody breast (WB) as a species (Sihvo et al., 2017). The WB has a predominant effect on the pectoralis major, although it may also have an effect on the pectoralis minor. At 2 weeks of age, it appears as a confined lesion in the pectoral muscles that grows into a fibrotic fracture with a hardened and pale look. The pectoralis major muscles of broiler chickens are affected by SM, which compromises their integrity (Petracci et al., 2019; Abasht et al., 2016).

Selection for faster growth is often linked to a number of skeletal abnormalities that affect locomotion to varying degrees. Lameness is a term that refers to a group of issues with movement caused by bone disorders. Epiphyseal ischaemic necrosis, tibial dyschondroplasia, epiphyseal division, valgus-varus deformity, skeletal fracturing, angular bone deformity, bent

knee, gastrocnemius tendon rupture, spondylolisthesis (kinky-back), and valgus-varus deformity are among the skeletal defects (Julian, 1998).

The only way to pick and raise chickens before the early 1900s was to find the right breeder for the phenotype of concern and mate them for the next generation (Siegel et al., 2006). However, poultry breeding technology has progressed since then. The technologies were designed to help with i) poultry reproduction management, (ii) pedigree monitoring, (iii) mating, and (iv) correct use of true breeding values of potential candidates (Muir & Aggrey, 2003). Prior to the 1940s, breeding procedures were exclusively geared on the production of purebred animals from purebred lineages. However, soon after the breeding programs were put in place, broiler farmers started combining and crossing specialized lines to create industrial processing animals with distinct breeding objectives (Muir & Aggrey, 2003).

Broiler genetic selection for increased development of birds with productive economic characteristics and high heritability has proven to be very effective (Bahmanimehr, 2012; Berri et al., 2001). According to Pollock's analysis of the major broiler breeder sector, a 1% improvement in the hatchability of an integrator capable of maintaining 15 million eggs laid might result in a \$30, 000/week rise (Pollock, 1999). Breeding programs with very precise breeding value calculations are now possible because of new technology on farms and better knowledge of the gene networks that influence the traits that animals produce.

Despite the successful accomplishment of the positive results of genetic selection, many undesirable, unexpected, and dramatic consequences also emerged. All of these repercussions involve animal welfare services. Broilers chosen for their success characteristics are more likely to have behavioural, physiological, and immunological problems. A net result of linkage and pleiotropy has been referred to as the genetic similarity between characteristics (Rauw et al.,

1998). Owing to a lack of independent division during meiosis, two or more similarly located loci in genes controlling various traits on the same chromosome are inherited together, resulting in genetic linkage. On the other hand, pleiotropy occurs as a single gene regulates a variety of phenotypic traits. Due to the reciprocal regulation of attachment and pleiotropy, the interrelated molecular and biochemical processes involved in the selection of developmental characteristics often appear as undesirable genetic consequences. For example, fast-growing broilers that have been selected for their larger bodies have been found to be predisposed to hyperphagia because of abnormalities in their hypothalamic satiety pathways (Rauw et al., 1998).

Finally, substantial gains in the domestic chicken chosen for development would not have occurred without adverse impacts on the birds' physiology. Parallel to the accumulation of success traits, a slew of catastrophic repercussions are becoming apparent. Selection for rapid growth increases carcass fat, which has a direct effect on leg lameness in broilers (Soller & Eitan, 1984), muscle consistency fluctuations due to changes in slaughter and development ages (Gous, 1986), and disease accumulation, such as sudden death syndrome (Gardiner et al., 1988), pulmonary hypertension (Julian, 1993), and negative influences (Emmerson, 1997).

The main advantage of the faster growth and development of broilers is their capacity to grow to high body weights within an incredibly short period. The freshly hatched broiler chick weighs 40 gm and will reach 4000 gm in 8 weeks (Wideman 2013). On the other hand, the broiler range for accelerated growth has contributed to an enormous rise in broiler meat yield and an overall boost in feed conversion rates. While selection for exponential growth has resulted in great economic gains, it has also resulted in the emergence of serious metabolic disorders. Heavy body weights followed by thin skeletal frames in an increasingly increased number of broilers have contributed to cardiovascular and musculoskeletal disorders (Riddell, 1992). The original

drawback of accelerated growth was the heavy fat accumulation in the carcass (Chambers, 1990). Fat deposition affects the consistency of the meat and reduces the overall selling price.

Broilers subjected to severe selection for body weight have increased body fat as a result of decreased lipolysis rates and increased insulin and glucagon concentrations in the plasma (Calabotta et al., 1985). The fertility of the birds is a primary area of interest and concern in broiler development. Not just in spontaneously mated flocks (Chambers, 1990), but also in centuries-old artificially inseminated broiler lines, fertility is adversely correlated with growth (Decuyper et al., 2003). It has been argued that selective breeding for economically valuable qualities has a harmful influence on secondary sexual attributes, desire, and male mating capacity, resulting in lower flock fertility (Pollock, 1999). Egg fertility is determined by the embryo's genotype, which is influenced by genetic and non-genetic influences inherited by both parents (male and female). Male sperm content characteristics, such as semen production, sperm motility, sperm metabolism, and the proportion of dead or dysfunctional cells are all affected by increased body weight (Wilson et al., 1979). Males who have leg difficulties may exhibit behaviors that obstruct efficient mating (Brillard, 2003). Increased body weight in females has an effect not only on the bird's reproductive activity and physiology, such as the presence of sperm storage tubules but also on egg production (Brillard, 2003). Also, the birds with a higher body weight grow more eggs than those picked with low body weight, the proportion of damaged eggs is higher in these birds (Anthony, 1998). Defective eggs, such as double yolk, soft-shelled, no shelled, and extra-calcified, are caused by ovulation and egg packaging being out of alignment (Dey, 2017). Another major complication triggered by accelerated growth is ascites, or pulmonary hypertension syndrome (PHS) (Al-Rubaye, 2013).

In addition to impairing reproductive efficiency selection of broilers for increased body weight results in lower antibody responses (Qureshi et al., 1998), and causes high death rates at a particular age (Havenstein et al., 2003). Rapid growth in broilers results in the inability of vital organs such as the heart and lungs to increase production in proportion to the birds' body mass and to perform their functions adequately to meet the demands of the birds' bodies. The emergence of idiopathic pulmonary arteriole hypertension (IPAH) or pulmonary hypertension syndrome is a symptom of the cardiovascular system's inability to support the increasing oxygen requirements in these birds (PHS). Ascites, or water bell, is a disease caused by PHS, which causes aggregation of edematous fluid in the abdominal cavity of broilers. The terms "PHS" and "ascites" are synonymous in poultry. The disorder is caused by both environmental and genetic causes, with heritabilities ranging from 0.1 to 0.7 (de Greef et al., 2001; Lubritz et al., 1995).

Changes in the metabolic properties of the birds' muscles make fast-growing broilers more susceptible to environmental stresses. Muscle properties including low glycolic fiber content, strong blood capillary flow, high oxidative and endurance capability, and smaller muscle fibers enable energy storage, which helps the organism cope with environmental stresses. The presence of a high proportion of glycolytic fast-twitch fibers and a low proportion of oxidative slow-twitch fibers in certain muscles of broilers bred for rapid development may suggest the birds' susceptibility to environmental stress (Mason, 2015).

Another serious health problem in fast-growing broilers is a mismatch between increased body mass and the rate of elongation of leg bones (such as femora and tibiae), putting the bones under increased mechanical stress and jeopardizing the skeleton's structural integrity (Wideman, 2016). This is connected with lameness development in these broilers. In all of these instances, excessive torque causes osteochondrotic clefts in the exposed growth plates' chondrocytes, where

an opportunist bacteria colonizes, resulting in bacterial chondronecrosis with osteomyelitis (BCO) (Wideman, 2016). The current dissertation aims to elucidate some of the aspects of BCO.

Skeletal inadequacy contributing to lameness is another issue linked to the fast growth of broilers. Broilers that are rapidly growing need more complex nutrition. Skeletal abnormalities are often missing or rare in broilers that mature slowly. Skeletal diseases are twice as common in male broilers as in females, which may be because males grow faster than females (Classen & Riddell, 1989).

### **The causes of lameness in broilers.**

The causes of lameness were classified into three groups: degenerative disorders, metabolic disorders, and developmental diseases (SCAHAW, 2000). Since then, lameness has been classified into five groups based on the pathogenic component that causes it: nutritional disorders, viral illnesses, conformational difficulties, metabolic abnormalities, and pollutants (Szafraniec et al., 2022).

Femoral head necrosis (FHN) was historically used to refer to necrotic lesions affecting the proximal femoral head, proximal femoral degeneration, or bacterial chondronecrosis (Bradshaw et al., 2002), but researchers changed the name to Bacterial chondronecrosis with osteomyelitis (BCO) after discovering that various points in the broiler skeleton, including the proximal femoral head, the proximal tibiotarsus and the fourth thoracic (T4) vertebra (with spondylitis) were also infected by different bacterial infections (McNamee et al., 1998). Additionally, osteomyelitis, proximal femoral degeneration, long bone necrosis, bacterial

chondronecrosis, and bacterial chondritis with osteomyelitis are BCO-related terms (Butterworth, 1999; Jiang et al., 2015).

In comparison to other methods of meat production, the chicken business has been fairly effective at producing marketable goods with reasonable turnaround times and little environmental impact. Regrettably, these efforts seem to be related with a variety of broiler health issues, one of which is lameness (Gocsik et al., 2017). Numerous factors, including genetic determinants, species, diet, sex, age, infectious agents, growth rate, and body weight, all played a role in the development of leg problems (Kierończyk et al., 2017). Trauma-related or mechanically induced difficulties, toxicity, feed conversion quality, as well as handling and transportation, are all contributing reasons (Aydin, 2018). Apart from these parameters, Knowles et al. (2008) emphasized the need of effective herd activities. If left unchecked, they exacerbate the problem of bone problems. Lameness may be generative, hereditary, or infectious, depending on the pathogen that produces the illness (Kierończyk et al., 2017; Fig. 1.9).

Birds' lameness is defined by a distortion or stiffness of the bone structure, which leads to the birds' partial or full immobility. The gait scale (GS), which ranges from 0 for normal to 5 for full immobility of the birds, can be used to quantify lameness (Kestin et al., 1992). According to some scholars, who cited high GS and irregular nociceptor thresholds, discomfort is linked to lameness and underlying pathologies (Nääs et al., 2009). Other researchers argue that there is no connection between lameness and discomfort (McNamee et al., 1998). Varus-valgus (VVD), tibia dyschondroplasia (TD), rickets, chondrodystrophy (Cook, 2000), spondylolisthesis (kinky back), epiphyseal detachment, broken gastrocnemius tendon (Julian, 1998), and touch dermatitis (Aydin, 2018) are examples of developmental deformities. Infectious infections are recognized as bone-damaging agents by the North Central Regional Committee (NC-187) (Cook, 2000). In

poultry, the skeletal system is not directly affected by bacteria, mycoplasma, and viruses, but they affect the joint gap (fluid accumulation) and soft tissues (tendons and synoviae), leading to severe symptoms of leg disorders. It might be difficult to determine the implicit etiology of non-infectious skeletal abnormalities since they are not mutually exclusive in the sense that they may interact with one another (Bradshaw et al., 2002). Despite the fact that the science of lameness and pain is still somewhat ambiguous, most poultry professionals believe that lameness in broiler chickens contributes significantly to animal health and welfare problems in the United States and elsewhere in the globe (Moura et al., 2006). Figure 1.8 shows several variables linked to the occurrence of lameness. Many factors lead to lameness, such as growth rate, biology, weight, activity, nutrition, husbandry, daily pattern of lighting (1 hour of darkness; 23 hour of light), age, sex, and contagious agents (Kestin et al., 2001; Reiter, 2006). In broiler chickens, BCO is the most common cause of lameness (Dinev, 2009; Al-Rubaye et al., 2015).

### **Pathogenesis of BCO lameness.**

Broilers can gain 8 pounds in 8 weeks (Wideman, 2016). This weight increase would be impossible to maintain without a corresponding increase in the size and intensity of the bird's skeletal structure. BCO has a perplexing etiology (Wideman et al., 2012). Rapid bone development is required for BCO pathogenesis because it imposes excessive mechanical pressures on the anatomically immature growth plates of the thoracic vertebrae and proximal leg bones (McNamee & Smyth, 2000), contributing to the development of clefts and microfractures within the physical (osteochondrosis) and epiphyseal cartilage (Wideman & Prisby, 2013). As a consequence of the truncation of the blood arteries that reach this location, tiny regions of localized ischemia and necrosis develop. In young broilers, long bone formation involves



elongation of growth plates at both ends of the bone shaft/diaphysis as well as diameter expansion as part of the complicated remodeling of the cortical bone (Wideman & Prisby, 2013). Wideman (2016), states that broilers are more vulnerable to lameness than layers since the former has a disproportionate weight gain ratio of skeletal structure maturation than the cranial-caudal redistribution of muscle mass. Rapidly developing birds had a higher rate of lameness and attempt to mitigate early development reduced broiler disease (Wideman, 2016).

During the same time span, growing broiler birds exhibit a four-fold rise in tibia and femur length, with a five-fold rise in mid-shaft diameter (Yair et al., 2012; Applegate & Lilburn, 2002). In certain species, osteochondrosis is caused by biophysical stressors and sluggish blood flow in the metaphyseal arteries. Bacteria may colonize pre-existing diseases in the affected areas, such as physical vascular disruptions, microfractures, and clefts, which provide entry points for opportunistic microbial infections (Thorp et al., 1993; Bond et al., 1991). Infectious hematogenous bacteria reach the bloodstream via the gastrointestinal tract or the respiratory system, where they can spread via the bloodstream, leave the circulation through the fenestrated epithelium at the ends of the physical and epiphyseal vascular plexuses, and travel through the cartilaginous matrix (Fig. 1.8a; Emslie & Nade, 1983; Wideman, 2016).

Bacterial translocation and growth are promoted by stress-induced immunosuppression (Wideman & Prisby, 2013). Bacterial emboli can form in the epiphyseal and metaphyseal circulatory plexuses when bacteria are translocated (Emslie & Nade, 1983; McNamee & Smyth, 2000). Plexuses are required for the survival and development of chondrocytes. Bacteria are known to attach to and colonize osteochondrotic clefts, as well as adhere to the exposed cartilage matrix. Pathogens can enter these locations, where they can grow in the absence of surveillance by antibiotics and circulating leukocytes (Emslie & Nade, 1983; Emslie et al., 1984). Because of

the lytic chemicals that bacteria emit when they settle in, terminal BCO is called necrotic degeneration.

BCO manifests itself macroscopically as centric patches of lytic zones or yellow caseous exudate that make afflicted bones weak and breakable (Al-Rubaye et al., 2013). BCO-associated lesions range in size from tiny pale spots at the growth plate to larger areas of yellow tissue extending from the growth plate to the medullary cavity (Fig. 1.8b; McNamee & Smyth, 2000). The proximal femur and tibiae can be classified into the following six diagnostic categories based on their macroscopic appearance: normal tibia head (normal tibial), tibial head necrosis (THN), extreme or caseous tibial head necrosis (THNsc), normal femoral head (normal femur), femoral head separation or epiphyseolysis (FHS), and femoral head nec (FHN). These classifications emphasize the severity of BCO lesions (Jiang et al., 2015; Fig. 1.8c). Due to the extensive necrotic abscesses and voids, BCO lesions seem to contain clusters of basophilic bacteria in the metaphyseal or epiphyseal blood vessels, which are covered by a weakly stained cartilaginous matrix (Wideman et al., 2014).

Wideman et al. (2012) have found a model of wire flooring for lameness induction in growing birds (Wideman et al., 2012). The model causes shear tension in fast-growing young birds, causing lameness with or without bacteria in the water (Wideman et al., 2013; Al-Rubaye et al., 2017). Several broiler product lines were tested on this wire-flooring system, and it was revealed that all of them, with the exception of a few, were sensitive to the occurrence of BCO-lameness (Wideman et al., 2014; Al-Rubaye et al., 2017). The starting point of BCO lameness seems to begin with mechanical micro-fracturing of insufficiently calcified columns of cartilage cells (chondrocytes) in the proximal growth plates of the femora and tibiae of early fast-growing young broilers (Wise, 1971; Riddell, 1983). The micro-fractures create osteochondrotic crypts,

which are inhabited by opportunistic bacteria that are spread throughout the body (Al-Rubaye et al., 2015; Mandal et al., 2016). The germs are spread vertically from broiler parent breeders to the chicks or horizontally from a contaminated hatchery or eggshells to the chicks (Stalker et al., 2010). Bacteria can enter the bloodstream of a chick through the gastrointestinal tract, respiratory system, or integumentary system (Wideman, 2016; Fig. 1.8a). Many terminal epiphyseal and physical vascular plexuses spread translocated bacteria hematogenously to all ends of the growth plate (Wideman & Prisby, 2013; Fig. 1.7). Investigating the anatomical makeup of the blood supply of broilers is critical as it affects the occurrence of lameness.

### **BCO lameness incidences.**

For economic and animal health reasons, the lameness caused by BCO is critical for the poultry industry. The most prevalent cause of lameness in broiler flocks is reported to be BCO (Wideman et al., 2012). It was initially identified in Australia as a cause of lameness in commercial broilers in which *Staphylococcus aureus* was isolated from lesions (McNamee & Smyth, 2000). According to a study of 20 broilers in Victoria, Australia (Wijesurendra et al., 2017), BCO affects broilers at a high rate throughout their lives, with various lesions diagnosed in around 28% of the birds. Following that, BCO was found in broilers from additional nations, including Canada, Australia, Europe, and the United States of America. Abnormalities of bone are found to be the primary cause of broiler mortality and losses in Canada (McNamee & Smyth, 2000). In the United Kingdom, FHN has been identified as the most common cause of lameness in broilers (Butterworth, 1999). As a consequence, it was shown that BCO is mostly responsible for leg difficulties. According to Bradshaw et al. (2002), BCO is now the most prominent cause of leg problems in commercial broiler farms worldwide. Long bone anomalies were identified as

the leading source of flock losses owing to lameness in Canada, accounting for 10% of total flock losses due to osteomyelitis and arthritis (Riddell & Springer, 1985). Riddell and Springer (1985) determined that the incidence of birds killed owing to lameness ranged from 0.46 to 4.08% based on data collected daily and from weekly necropsy examinations of chickens from 51 broiler flocks in Western Canada (Riddell & Springer, 1985). In Northern Ireland, research was carried out to determine the presence of BCO in commercial broilers (McNamee, 1998). 28 male broiler flocks and 19 female broiler flocks were studied, and 0.38 percent of female flocks and 0.52 percent of male flocks were determined to be lame. Additionally, McNamee et al. discovered BCO in 17.3 percent of culled chickens with leg abnormalities and 0.52 percent of all broilers culled with leg problems (McNamee et al., 1999).

An investigation of broilers in the United Kingdom, Italy, and France revealed a 16% incidence of lameness (Bassler et al., 2013). In a post-mortem investigation, FHN and BCO were discovered to be the most prevalent causes of lameness in commercial broilers, accounting for 38% of all cases, which was followed by hock infection (13.1%), twisted leg 11.1%, and lastly, tibial dyschondroplasia (TD) 7.2% (Pattison, 1992). Another research found that lameness was prevalent in 0.38 percent of female broilers and 0.52 percent of male broilers. According to the UK chicken association, a study of 176 commercial broilers revealed that only 27% had restricted movement and just 3% were categorized as lame (Knowles et al., 2008). In recent years, the Farm Animal Welfare Council of BCO has gradually recognized osteomyelitis as the primary cause of lameness in commercial broilers (Council & Britain, 1992). Various names for this disease have been documented in the past, including proximal femoral degeneration, long bone necrosis, femoral head necrosis (FHN), and osteomyelitis. While other classification systems are used, BCO is considered the most appropriate one because it describes the pathology

accurately, including necrotic degeneration, and also shows microbial infection involvement, which is more common in the proximal head of the femur and tibia, and T4 vertebra, as examples of other rapidly growing bones. Osteomyelitis is considered a prevalent condition in poultry, such as chickens, ducks, turkeys, and other species (Wise, 1975).

A typical epidemic outbreak of BCO lameness results in the loss of 1.5% of broilers from infected flocks. However, this ratio might be even higher. BCO epidemics can result in the loss of more than 15% of a flock (Rebello, 2019). In research (McNamee & Smyth, 2000), an estimated 0.75% of all birds were found to have lameness attributable to bacterial chondronecrosis with osteomyelitis (BCO). Broilers with bone disorders had a 1.72 percent average incidence, with 1.1 percent being culled in the field and 0.62 percent being condemned. It presents as in broilers as necrotic degeneration and bacterial infection, which occur mostly in the birds' rapidly growing bones and joints (McNamee & Smyth, 2000). According to the findings of a study conducted on 67 lame birds to determine the prevalence of BCO in the proximal femurs, 64% of the birds had gross degeneration of the femoral end, which was primarily caused by osteomyelitis; 25% had epiphysiolysis, and the microscopic bacterial or osteochondritis lesions were found in the remaining 11% (Thorp et al., 1993). At least 15% of the mortality in broilers in Bulgaria was due to lameness, and in 90% of these cases, BCO was the primary cause (Wijesurendra et al., 2017).

In the last 20 years, more than 1.5% of commercial broiler flocks reared to processing weights at 5-8 weeks in the United States may have been afflicted by spontaneous BCO and lameness (Stalker et al., 2010; Wideman & Prisby, 2013). Since BCO was associated with a low prevalence of lameness in poultry, numerous experimental models were developed to investigate the pathology and source of BCO (McNamee et al., 1999). The bulk of these models use the

intravenous injection, aerosol inhalation, or tracheal administration of common pathogenic agents to birds. Additionally, a newly developed wire flooring model was used to produce significant BCO levels, which will assist in the systematic and correct study of the disease's origin (Gilley et al., 2014). In the experiment, wire flooring was used instead of wood shavings as litter to keep young broilers growing quickly and moving around at a normal level of activity. This was carried out to apply torque and shear stress on weak proximal leg joints at low stocking densities, which is what happens in commercial systems.

### **The impact of BCO lameness on the economy.**

Healthy broilers develop at a faster rate than lame ones (Yalcin et al., 1998; McGeown et al., 1999; Manohar et al., 2015). According to estimates, broiler lameness triggered by FHN (femoral head necrosis) is expected to cost the UK broiler sector £4.7 million per year (Butterworth, 1999). Another study done in the United Kingdom discovered that 61% of birds with FHN also had indications of bacterial infection (Thorp, 1994). Additionally, expenditures and losses were \$120 million in the United States alone as a result of these growth interruptions and improvements in the area of musculoskeletal systems in the chicken industry. Skeletal abnormalities were shown to have an indirect influence on profitability as a consequence of increased broiler meat processing, resulting in a 10–40% decrease in total profit (Aydin, 2018). Male broiler lameness caused by FHN is estimated to cost the Northern Ireland broiler industry £185, 625 per year, whereas female broiler lameness is estimated to cost the Northern Ireland broiler industry £118,000 per year (McNamee et al., 1998). Over the previous 70 years, the broiler market has evolved dramatically; from small-scale poultry farms to a system that is more strongly integrated and industrialized, and is controlled by a few large corporations, and

currently more than 20 billion broiler chicks are processed yearly (Lowder et al., 2009).

Lameness leads in a drop in chicken earnings for poultry breeders. This is attributable to higher death rates, culling of lame birds at various production phases, and the birds' condemnation throughout the process.

Poor growth, greater condemnation, and downgrading at slaughter are significant economic losses for broiler flocks that are afflicted (McNamee & Smyth, 2000). Due to the rise in body weight, bone deformities have also grown. Lameness is a major contributor to broiler morbidity and mortality. Because of this, there is a negative correlation between the degree of lameness and the ultimate body weight (Gocsik et al., 2017).

As a result of the detrimental effect of lameness on productivity, the overall performance of the poultry industry, including production costs and returns, has declined (Gocsik et al., 2017). For the poultry industry, leg problems are projected to cost hundreds of millions of dollars yearly (Bradshaw et al., 2002). Numerous studies have showed that lame birds with severe lameness endure pain when walking, making important functions such as reaching for water and food more difficult (Caplen et al., 2014). Each year, around 12.5 billion birds are estimated to be afflicted by leg disorders. The yearly cost of leg disorders in the broiler business is \$120 million. These diseases include valgus-varus abnormalities, tibial dyschondroplasia, femoral head necrosis, and rickets (Cook, 2000). Lame birds lose weight because they are less willing to walk to feeders, which results in lower food intake (Yalcin et al., 1998).

The Farm model estimates the economic impact of lameness by taking into account a greater condemnation rates at slaughter, increasing feed conversion, increased mortality, and decreased weight gain (Gocsik et al., 2017). The Farm Model estimates the economic burden of lameness as a function of the frequency of lameness in birds with  $GS \geq 3$  and its effect on the

productivity of poultry. To determine chicken production costs, net profit per kilogram of delivered broiler, production costs, and gross margin (revenues minus variable expenditures) are all needed (Gocsik et al., 2017). The chicken industry in the United States of America loses around \$100 million each year for a variety of causes, or about \$.016 per broiler. This has an influence on production costs and, eventually, on the price of chicken products (Aydin, 2018). Broiler breeders might be able to lessen the negative effects on the economy caused by leg weakness and lameness by identifying risk factors and implementing appropriate management strategies to keep them under control (Knowles et al., 2008).

### **Disorders in chicken leg.**

Broilers with less physical activity are more likely to be lame (Dawson et al., 2021). The broilers with lameness are sleeping and resting more than the healthy birds (Wideman, 2016). Among agricultural animals, broiler chickens are the quickest growing and cheapest source of protein (Beski et al., 2015). Broilers with a rapid development rate and enhanced meat yields were developed via intensive selection processes. This results in aberrant body part growth, such as early muscle development without parallel skeletal development, resulting in problems and limb weakness (Aydin, 2018). Numerous variables affect the general integrity of the skeletal system, including the environment, locomotive activity, development rate, age, contagious diseases, diet, management, genetics, and toxins (Rath et al., 2000).

Normal walking ability can be defined as a feature of the skeletal, muscular, and nervous systems that are interconnected (Butterworth, 1999). As a consequence, if any of these organs fails, lameness or impaired leg function will occur. Leg weakness, lameness, and other bone



disorders in broilers are severe issues for animal welfare and productivity, resulting in increased morbidity and mortality (Dawkins & Layton, 2012).

Following the objectives of the breeding program in the production of the greatest muscle mass in broilers, there is an inverse association between the rise in the mass of the wing muscle and a decrease in the leg muscular perimeter, resulting in unfavorable effects on young birds' performance and well-being (Rath et al., 1999). Additionally, the physical fragility associated with pectoral growth results in cranial displacement of the avian center of mass, impairing the leg's ability to maintain a sustainable balance while carrying the whole body weight (Nääs et al., 2009). The buildup of more muscle mass and changes in body form produced by a shifted mass center and shorter leg length place enormous strain on the broiler's young skeleton. As a result, these structural alterations are highly related to deformities, such as weakness, deformation, infestation, osteoporosis, and contusion. This implies that a rising number of industrial broiler bodies are not developing in harmony with these features, perhaps putting them at risk of being lame (Kierończyk et al., 2017). The lying downtime of broilers has risen from roughly 76% in normal birds to 86% in lame ones. In cases of severe lameness, the amount of time spent walking at a processing weight is cut from 3.3% to 1.5% as broiler weight is linked to a higher chance of lameness.

### **Key BCO bacteria.**

Broiler lameness may be caused by a variety of factors. However, there are two main types of lameness triggers: contagious causes that trigger extreme lameness and skeletal disorders that cause less severe lameness (Thorp et al., 1993). While the specific etiology of BCO lameness is unknown, it is suspected that bacteria play a key part in the disease's spread. A diverse spectrum of opportunistic bacteria has been implicated in the development of BCO, according to the literature (Nairn & Watson, 1972).

A number of bacterial species, including *Escherichia coli*, and *Staphylococcus* spp., *Staphylococcus aureus*, have been recovered from BCO lesions, and these bacteria are commonly seen in mixed cultures with other bacteria, such as *Salmonella* spp. Furthermore, pure cultures of *Enterococcus cecorum* were recovered from osteomyelitis lesions in broilers in Scotland and the United States (Armour et al., 2011). At the University of Arkansas chicken research farm, *Staphylococcus agnetis* was found to be overrepresented in BCO lesions from lame broilers (Al-Rubaye, 2013). These bacterial strains were isolated from 81 (87%) out of a total of 93 samples tested. This result was not unexpected. However, the discovery of *S. agnetis*, which had previously been linked to mastitis in cows but had not previously been linked to broiler chickens, was unexpected. Microfractures and osteochondritic breaks in the bone are caused by the unsupported weight of rapidly increasing birds. The cracks contain exposed collagen matrices that may provide favorable conditions for the inhabitation and colonization of opportunistic bacteria from a variety of sources that are hematogenously disseminated (Wideman et al., 2012).

In addition, the arteries that feed blood to the femur, tibia, and vertebra become narrower, forming capillaries. These capillaries are nets of perforated endothelium large enough to allow

the transfer of certain blood components, such as bacteria, into the joint cartilage matrix (Wideman et al., 2012). Epiphyseal and metaphyseal blood arteries are obstructed by translocated bacteria that have clung to exposed collagen complexes. Wideman and Prisby (2013) reported that the obstruction helps bacteria form foci and stops pathogens or antibiotics from interfering with the reaction (Wideman & Prisby, 2013).

The crypt morphology and richness of the intestinal villus in chickens are connected with the natural microbiota of the chicken (Mandal et al., 2016). The macroscopic pathology and villus length in lameness were evaluated, and it was discovered that probiotic treatment improved villus length more than the control group (Alrubaye et al., 2020). An abundance of short-chain fatty acids produced by the gut microbiota encourages broiler growth. Additionally, it is an important factor in detoxification, immune system control, polysaccharide metabolism, nutrition absorption, and overall bird health (Yeoman et al., 2012). The microbiome of the gut or other tissues that are important in lameness and other diseases is not well described or understood (Jiang et al., 2015). Nonetheless, the majority of chicken microbiota investigations indicate a preference for the stomach over the trachea, feces, or blood, with the bones seeming to be the least frequent (Mandal et al., 2016). Jiang et al. (2015) hypothesized that a variety of bacterial species move mostly from the gut community into the circulation system, developing niches in various organs. Despite being aware of the microbial communities present in the yolk remnants and respiratory tracts of normally healthy birds, these researchers underlined the significance of gut microorganisms (Jiang et al., 2015).

A variety of opportunistic bacteria have been identified from BCO lesions, including *E. cecorum*, *Staphylococcus* spp., *Salmonella* spp., and *E. coli* (Al-Rubaye et al., 2017; Mandal et al., 2016; Wijesurendra et al., 2017). While it is crucial to describe BCO isolates in

order to ascertain their influence on the incidence of lameness, we must also understand their origin and route of spread. Bacteria essential for BCO transmission may be conveyed through the respiratory system, the skin surface, or the gut microbiome, as seen in Figure 1.7a (Wideman, 2016). As a result, it is critical to investigate the microbial communities of broiler chickens and the role they play in the BCO infection process. It would be necessary to investigate the mechanisms used by opportunistic bacteria to elude detection by the immune system before pathogenicity and how the host immune system might be reinforced.

Knowing the dispersion of microorganisms may help us understand the pathways and circumstances required for bacterial invasion, which are crucial in the development of BCO and other dysbiosis-associated disorders. Clavijo & Flórez, (2018) conducted an investigation into the relocation of microbial communities throughout the tissues of the intestinal system and discovered that the taxonomic profiles reported for various portions of this system are different. Food, sex, biology, antibiotic usage, and sample methodologies all have a role. Numerous species of the genus *Lactobacillus* and the family *Clostridiaceae* are found in chicken gizzards and crops. The crop conditions encourage bacteria to metabolize starch and ferment lactate. Gastric fluids, pepsin, and hydrochloric acid acidify the environment in the gizzard, reducing the amount of fermentation and overall bacterial growth. *Lactobacillus*, *Enterococcus*, and *Clostridiaceae* make up the majority of the bacterial cell count in the small intestine (Clavijo & Flórez, 2018). Clavijo & Flórez (2018) also stated that the ceca are also ranked as one of the most diverse areas in terms of species because the ceca can retain food for up to 20 hours and help reabsorb and concentrate urea, and it is also known to be a site for fermentation of undigested carbohydrates in the intestines. Among other groups of bacteria, the ceca are abundant in Phyla *Bacteroides*, *Firmicutes*, *Clostridiaceae*, and *Proteobacteria*.

In 2016, Mandal et al. (2016) collected blood samples from 240 healthy birds and 12 lame birds in an attempt to characterize tissue (including gut) microbiomes. Mandal et al. (2016) used deep sequencing and analysis of bacterial 16S rRNA sequences to describe tissues, including gut microbiomes. Mandal et al. (2016) found that Bacteroidetes accounted for 14%, Proteobacteria 60%, Firmicutes 11%, Actinobacteria 10%, and Cyanobacteria accounted for 97% of the phylum level communities in chicken blood (Mandal et al., 2016). These characterizations were established after a study of around 40 operational taxonomic units (OTUs) independent of age, host physiology, or environmental variables. Mandal et al. (2016) found that the population of *Microbacterium*, *Staphylococcus*, and *Granulicatella* in lame bird blood was much higher than that in healthy bird blood (Mandal et al., 2016).

According to Jiang et al., the phyla of Proteobacteria predominated with 91% of the population, Firmicutes with 6%, and Actinobacteria with 2%. Additionally, several other phyla were represented in minor quantities, including Acidobacteria, Bacteroidetes, Cyanobacteria, Nitrospirae, Tenericutes, and Verrucomicrobia, which all constituted less than 0.4 percent of the total phylum. *Staphylococcus* spp. species were the most overrepresented (Jiang et al., 2015). Several *Staphylococcus* species have been isolated from birds with BCO on our facility and commercial farms that are diagnosed with *Staphylococcus* (Shwani et al., 2020; Ekesi, 2020). Jiang et al. came to the conclusion that a reduction in species variety is connected with a greater degree of lameness and BCO lesions (Jiang et al., 2015). To better understand why chickens become lame and how to treat it, more research is needed into the BCO microbial communities of broiler chickens and how they play a role in the BCO infection process.

Moreover, firmicutes are a phylum that has a large number of microorganisms with unknown phylotypes, which makes this phylum of great interest to researchers. Taxa including

*Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli*, *Clostridium perfringens*, and *Salmonella enterica*, which may be detrimental to chickens and humans, are found in the gut microbiome of chickens (Sergeant et al., 2014). *Campylobacter* spp. is considered harmful to humans, but not to birds. *Salmonella enterica* may be lethal to birds, depending on their age, the *Salmonella* spp. serotype, and overall health. *Salmonella* species are more prevalent in lame birds. *Escherichia coli* maintains a modest incidence in chicken intestines throughout the lifespan of apparently healthy chicks. APEC *E. coli* contains a number of significant virulence factors that are required for the development of a variety of avian illnesses (Clavijo & Flórez, 2018). Jiang et al. (2015) looked at the microbiota of 97 tibial or femoral bones from healthy and lame broilers of different ages, lines, lesions, and floor types to learn more about how the long bones are linked to BCO.

### **Microbiology of the key BCO bacteria.**

A broad range of opportunistic bacteria was found to be associated with the incidence of BCO (Nairn & Watson, 1972). Typically, these bacteria are identified in mixed cultures with other bacteria, such as *Salmonella* spp., that have been associated with BCO (Andreasen et al., 1993; Joiner et al., 2005). Moreover, in the Scotland, and the United States, as well as from broiler breeders in the Netherlands, *E. cecorum* pure cultures were recovered from lesions of broiler osteomyelitis (Armour et al., 2011). The mechanisms of pathogenesis of each bacterium mentioned below may be essential in the occurrence of BCO and lameness in chickens, yet, they are not limited to chickens only.

### ***Staphylococcus* spp.**

*Staphylococcus* species are harmful and can be found in a variety of environments, including the air, water, and soil. They are regarded as part of the natural flora of the chicken skin and mucous membranes, and they are widely spread throughout hatcheries, chicken houses, and processing factories, amongst other places. Infection with any *Staphylococcus* species can cause disease. Additionally, members of this genus have been found in a range of animal taxonomic groupings, including poultry. At the moment, this genus has more than 70 species. Around 60 *Staphylococcus* species have been found in broilers (Szafraniec et al., 2022). Lameness caused by BCO is often caused by bacteria belonging to the genus *Staphylococcus* (Bradshaw et al., 2002). The virulence factors vary across the genomes of species and strains of the *Staphylococcus* genus. In broilers infected by BCO, there have been concomitant losses owing to septicemia induced by *Staphylococcus* spp. in broilers (McNamee et al., 1998). Coagulase-positive bacteria, such as *S. hyicus*, and coagulase-negative bacteria, such as *Staphylococcus* spp., *S. simulans*, and *S. xylosus*, *Mycobacterium avium*, *E. coli*, *Enterococcus* spp., and *Salmonella* spp., are among the bacteria that can cause lameness and be isolated from infected bones of broilers (Reece, 1992). From 22 percent, 11.1 percent, and 13.3 percent, respectively, of broiler proximal femoral heads diagnosed with BCO, mixtures of coagulase positive and negative *Staphylococcus* species and *E. coli* were detected (Thorp et al., 1993). According to Riddell (1997), the bacteria *Staphylococcus* spp. was found to be the most often isolated from arthritis, tendinitis, and osteomyelitis in broilers from western Canada (Riddell, 1997). The species below are the common BCO associated *Staphylococcus* species.

### ***Staphylococcus aureus.***

*Staphylococcus aureus* is a Gram-positive coccus bacteria that develops in grape-like clusters. *S. aureus* is not motile, does not produce spores, is facultatively anaerobic, oxidase-negative, and catalase-positive. Cell-bound clumping factors are present in *S. aureus*. It is only marginally resistant to the effects of sodium chloride. *S. aureus* produces hyaluronidase as a result of the fermentation of mannitol. The physical appearance may change depending on the medium. It is unknown why *S. aureus* infects birds at a higher incidence of BCO and lameness than any other known BCO isolate. However, it is considered to be connected to its propensity to induce greater lameness. *S. aureus* does have the intrinsic potential to cause harm (McNamee et al., 2000). Many strains of *S. aureus* were recovered from the skin, nostrils of birds, the plantar and dorsal surfaces of the feet of normal chickens as well as wild birds (Harry, 1967; Cooper & Needham, 1976). As a result, wild birds are regarded as reservoirs of *S. aureus* with the possibility of transmission to broiler chickens. In addition, poultry house air, feeders, litter, and drinks could act as potential sources of *S. aureus* (Sauter et al., 1981).

*S. aureus* generates surface proteins that are required for both pathogenicity and adherence to host cells. Only a handful of the virulence proteins generated by *S. aureus* have been identified: fibronectin binding protein, protein A, fibrinogen binding protein, collagen-binding protein, and bone sialoprotein (Foster et al., 2014). In contrast to the common BCO isolate *S. epidermidis*, *S. aureus* has 18 distinct genomic islands, each of which contains virulence genes that compromise host defenses (Foster, 2012). While *S. aureus* produces coagulase, which differentiates it from other *Staphylococcal* species, the coagulase gene (*coa*) is not associated with disease (Crossley & Archer, 2009).



According to researchers, *S. aureus* is the most virulent and predominant staphylococcal species, which was observed to be the most frequently recovered pathogen from broiler leg and joint lesions, and *S. aureus* is the causal agent of childhood osteomyelitis and community-acquired infections as well as hospital-acquired infections in humans (Randall, 1996; McNamee et al., 2000). *S. aureus* is responsible for a significant proportion of mortality and morbidity in both people and domesticated animals due to a wide range of acute and chronic infections. Although the particular role of these capsules in bone and joint infection is unknown, it has been proposed that they help in the adherence of chicken cartilage (McNamee et al., 2000). The most common cause of lameness in commercial broilers diagnosed with BCO is *Staphylococcus aureus*, according to a broad panel of researchers (McNamee & Smyth, 2000; Al-Rubaye et al., 2017). *S. aureus* is usually connected with bovine mastitis or intramammary infection in dairy cattle, colonizes skin or mucosal epithelium, persists in the dairy environment (Wang et al., 2022).

Osteomyelitis, bumblefoot, arthritis/synovitis, omphalitis, and acute septicemia are all infections caused by *S. aureus*. Additionally, *S. aureus* is the predominant bacteria responsible for septicaemic epidemics linked with BCO infection (Emslie & Nade, 1983; McNamee et al., 1999). For many decades and in a wide variety of geographic locales, *S. aureus* has been commonly isolated from BCO (Wijesurendra et al., 2017). In an experiment carried out on 38 bones from broilers diagnosed with BCO, *S. aureus* was recovered in 63.1% of the bones, non-hemolytic *E. coli* was found in 13.1%, *S. xylosus* in 10.5%, *S. hyicus* in 10.5%, and *S. simulans* in 2.6%. (McNamee, 1998).

*S. aureus* was used to experimentally cause BCO in hens, with the bacterium being administered through intravenous injection (Emslie & Nade, 1983; McNamee et al., 1999). *S.*

*aureus* is deposited mostly at the developing ends of metaphyseal blood vessels, rather than inside the blood vessels themselves, in this situation. It has also been shown that exposing broiler chickens to *S. aureus* suspension through an aerosol boosts BCO, with greater rates among those infected with infectious bursal disease virus (IBDV) and chicken anemia virus (CAV; McNamee et al., 1999).

Some additional types of *Staphylococcus* have also been observed in poultry infections. The other most often seen species in poultry include *S. xylosus*, *S. cohnii*, and *S. lentus* (Zaki, 2020). *S. agnetis* was recently identified as the major septicaemic pathogen in broilers, causing BCO of the tibiae and proximal femora (Al-Rubaye et al., 2015). According to previous research, *S. agnetis* has been linked to both clinical and mild clinical mastitis in cattle (Adkins et al., 2017; Taponen et al., 2012).

### ***Staphylococcus agnetis.***

The *S. agnetis* species' namesake is Agnes Sjöberg (1888–1964), the European continent's first female veterinary surgeon. On bovine blood agar, it has the characteristics of being light grey, spherical, smooth, non-hemolytic, and opaque. *S. agnetis* cells are oxidase-negative, catalase-positive, facultatively anaerobic cocci that do not produce spores and do not move. They may grow singly, in pairs, or in tiny aggregates. *S. agnetis* is a Gram-positive staining bacterium that also has varied coagulase activity, and generally, it is coagulase-negative after 4 hours, but over 25% of the isolates are coagulase-positive after 24 hours, which is a significant number (Taponen et al., 2012). Bacterial colonies may have grown to a size of 3 mm after 24 hours of incubation at 37 °C. *S. agnetis* cells are not susceptible to enzymes such as lysozyme, polymyxins, and deferoxamine, unlike lysostaphin and novobiocin, to which *S. agnetis* cells are

not resistant. *S. agnetis* branches out from the *Staphylococcus* genus according to 16S rRNA sequence analysis, several housekeeping genes, such as *rpoB*, and *tuf*, or DNA fingerprinting employing amplified fragment length polymorphism (Al-Rubaye et al., 2015; Adkins et al., 2018).

*S. agnetis* is a frequent pathogen that has been identified from milk samples collected from the mammary glands of cattle with intramammary infections, in which subclinical or mild clinical mastitis in dairy cattle is the outcome of it. Additionally, *S. agnetis* has been shown to be related to lameness in broilers, and it has also been recorded to be collected from instances of septicemia and endocarditis in broilers (Taponen et al., 2012). Additionally, it was revealed that *S. agnetis* is overrepresented in BCO lesions detected on our Arkansas chicken research farm (Al-Rubaye, 2013), in which *S. agnetis* was isolated in 81 out of 93 (87%) samples. For the first time, this bacterium, *S. agnetis*, was linked to mastitis in cattle, which had not been previously linked to broiler chickens. As well, *S. agnetis* has been found as a component of the pig microbiota, specifically the ventral skin and vaginal microbiota (Kiefer et al., 2021). *S. agnetis*, along with other coccal species such as *S. aureus*, *S. lentus*, *Enterococcus faecalis*, *E. hirae*, and *S. simulans*, was isolated from the blood of broiler breeders who had footpad dermatitis or footpad sores and was linked to fatal infections (Szafraniec et al., 2020). In Japan, *S. argenteus* is abundant in retail food establishments as well as poultry slaughterhouses (Wakabayashi et al., 2022).

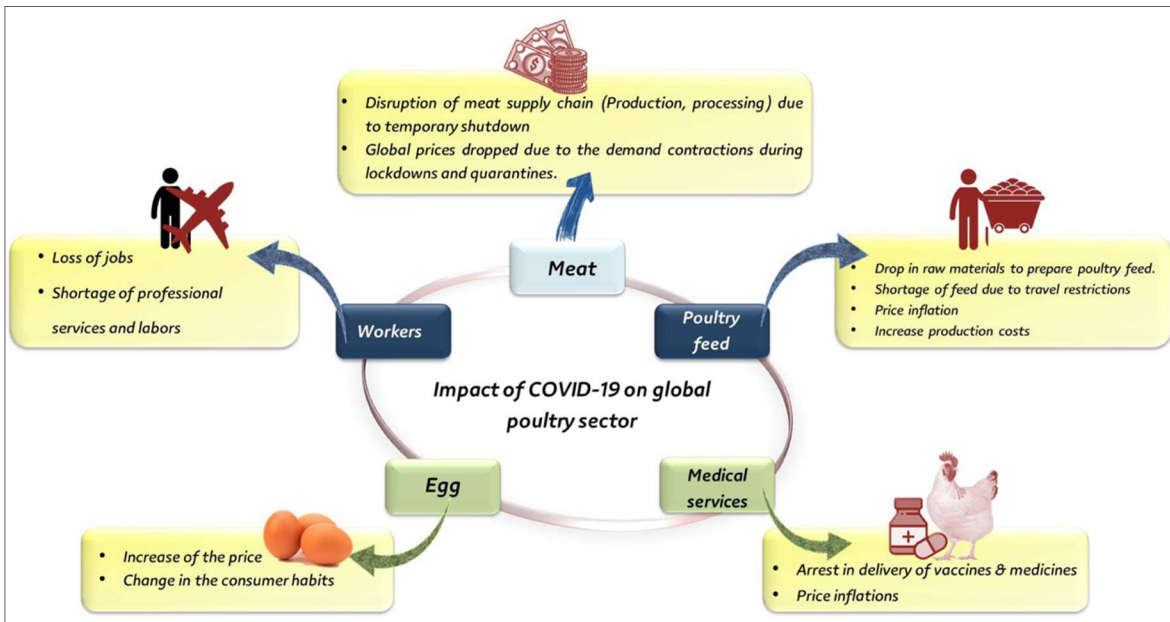
*S. hyicus* and *S. agnetis* are two species that are closely related to *S. chromogenes* and have both been found in BCO lameness. It has been found that *S. hyicus* is connected with osteomyelitis in Turkey and that it is also related to osteomyelitis in broilers (Szafraniec et al., 2020).

### **Virulence of *S. agnetis*.**

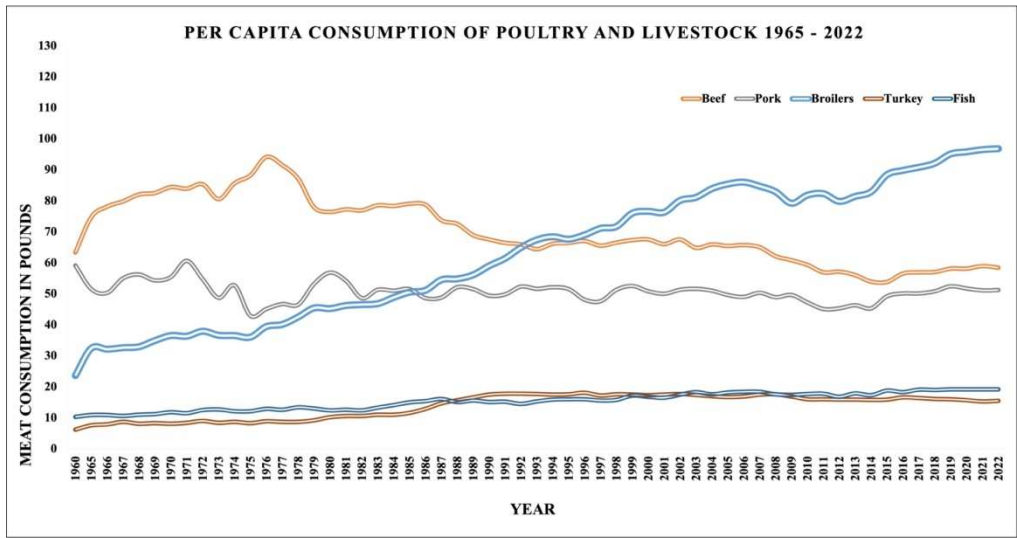
Being aware of the specific pathogen and the processes by which this pathogen is connected to creating the disease can aid in the development of measures for reducing BCO and , ultimately, reduce economic loss (Wideman, 2015). *Staphylococcus* spp. has been identified as the most frequently detected bacteria causing lameness in broilers, with *S. agnetis* being the most frequently detected strain (Wideman, 2012; Al-Rubaye et al., 2015). It has also been noted that bacteremia, or the presence of bacteria in the blood of lame and otherwise healthy broilers, may occur. Salpingitis, peritonitis, arthritis, septicemia, amyloidosis, endocarditis, and pododermatitis are some of the diseases that may result from a damaged epithelial lining of the footpads, which enables *Staphylococcus* species to enter the host cells and multiply. Based on the isolation of *S. agnetis* from blood samples, it seems that this bacterium species has spread to different organs and tissues in the body. A study was conducted on the University of Arkansas research farm to investigate the prevalence and pathogenicity of the chicken isolate *S. agnetis* 908, which was obtained from BCO lesions.. Broilers were given bacteria in their drinking water, which they then drank. *S. agnetis* has shifted from cows to broilers, and the many means of transmission comprise birds, hatchery staff, feed, the air, and flies. During challenging broilers with *S. agnetis* 908, it seems that transmission to pen mates happens by direct touch, nipple waterers becoming contaminated by exposed broilers, or shedding from infected birds (Al-Rubaye et al., 2017). Furthermore, the data show that *S. agnetis* 908 is likely to spread from the stomach into the circulation and establish a foothold in the proximal leg bones' rapidly developing growth plate.

The research was carried out to determine if *S. agnetis* was linked with mastitis, in which it was found that *S. agnetis* isolates have the capacity to internalize into bovine mammary

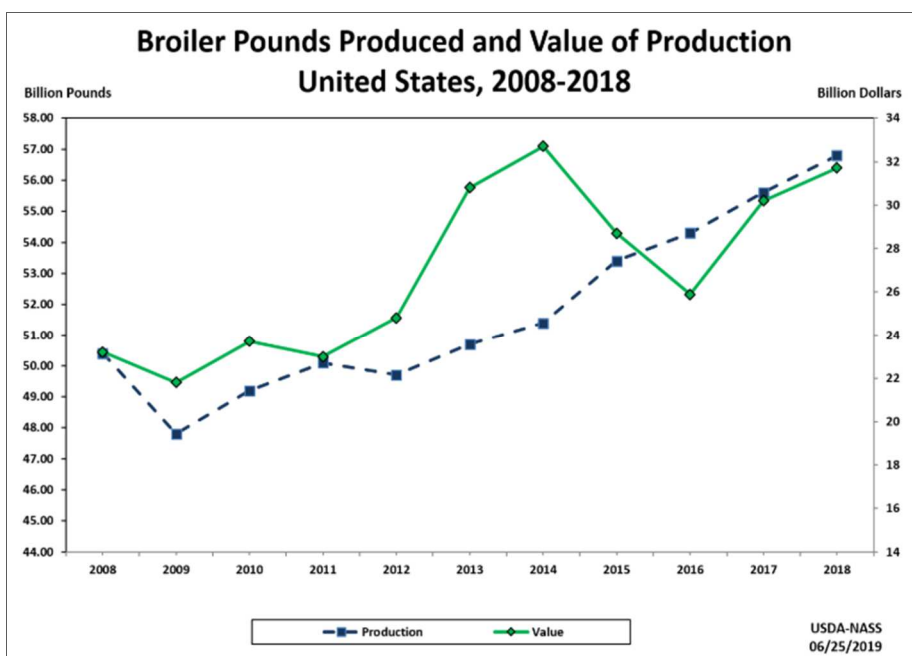
epithelial cells. This microorganism may enter host cells by receptor-mediated endocytosis, commonly known as the zipper mechanism (Chávez et al., 2019).



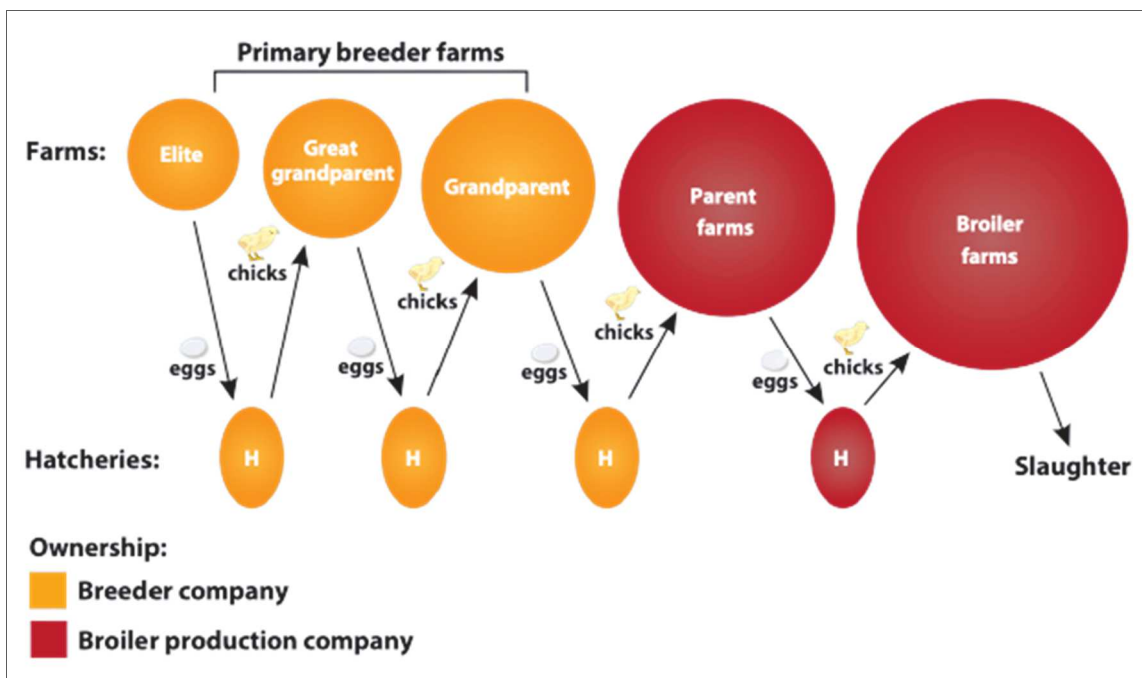
**Figure 1.1.** The worldwide effect of the COVID-19 epidemic on the poultry industry. (Copied from Attia et al., 2022).



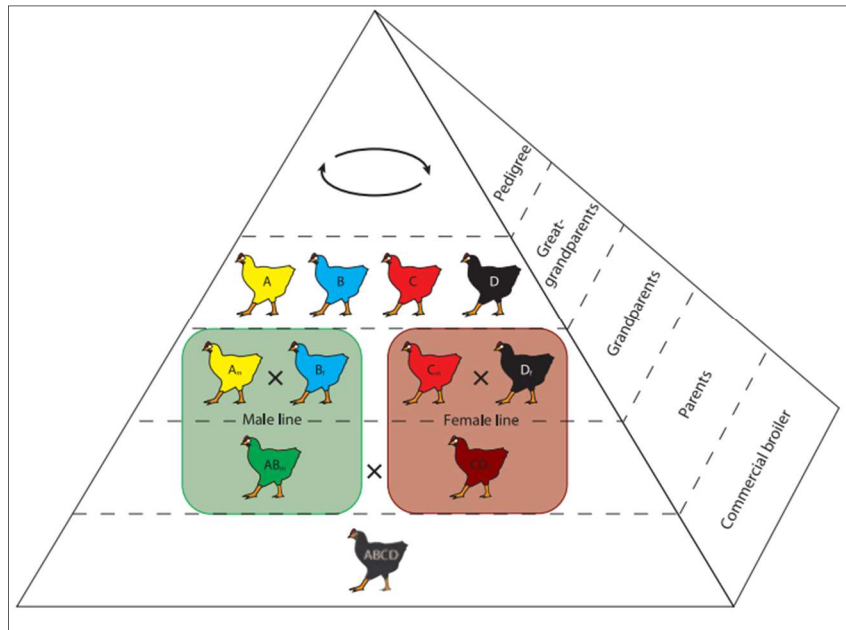
**Figure 1.2.** Broilers have been produced by the pound in the USA since 1965, according to a USDA report (Image copied from USDA, 2022).



**Figure 1.3.** From 2008 to 2018, US broiler production in pounds with an estimated monetary value (Image copied from USDA, 2019).

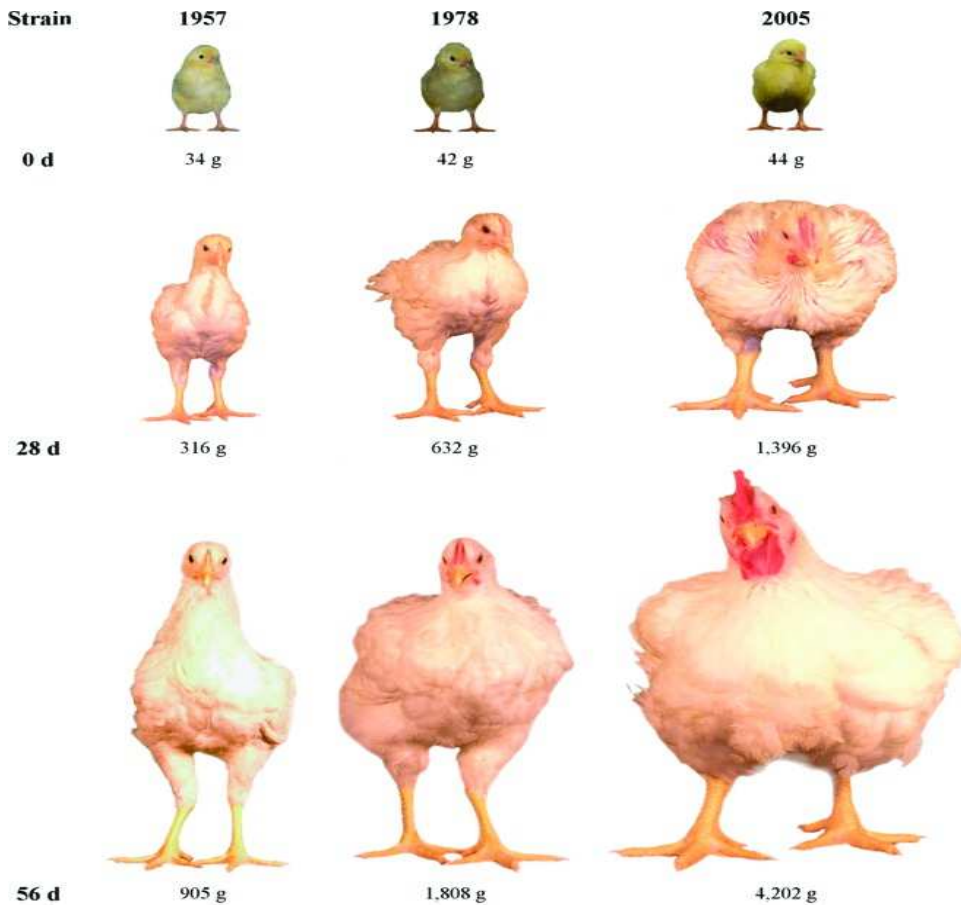


**Figure 1.4.** Main breeders, parent breeders, and commercial broilers are all types of broilers. Every generation's eggs are hatched to create the next generation's flocks. Broilers are essentially the product of multipliers in the parent line. Image copied from (USDA, 2013).

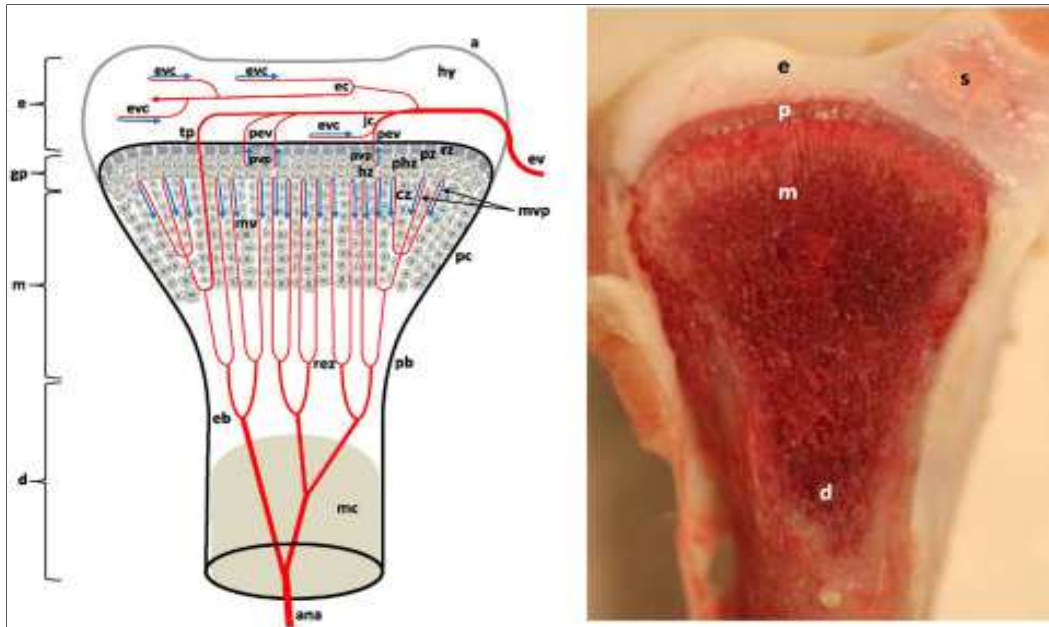


**Figure 1.5.** Generation and multiplication scheme in modern broiler development from elite/pedigree to commercial broiler products: This pyramid depicts a typical commercial broiler chicken breeding program, with each segment representing a generation responsible for transmitting selection response from purebred lines (at the top of the production line, where the selection of economically important traits occurs and is maintained) to billions of commercial broiler descendants (the fifth generation). The period span between pure line selection and commercial broiler gains is usually about 4.5 years (Copied from Paxton, et al, 2010).

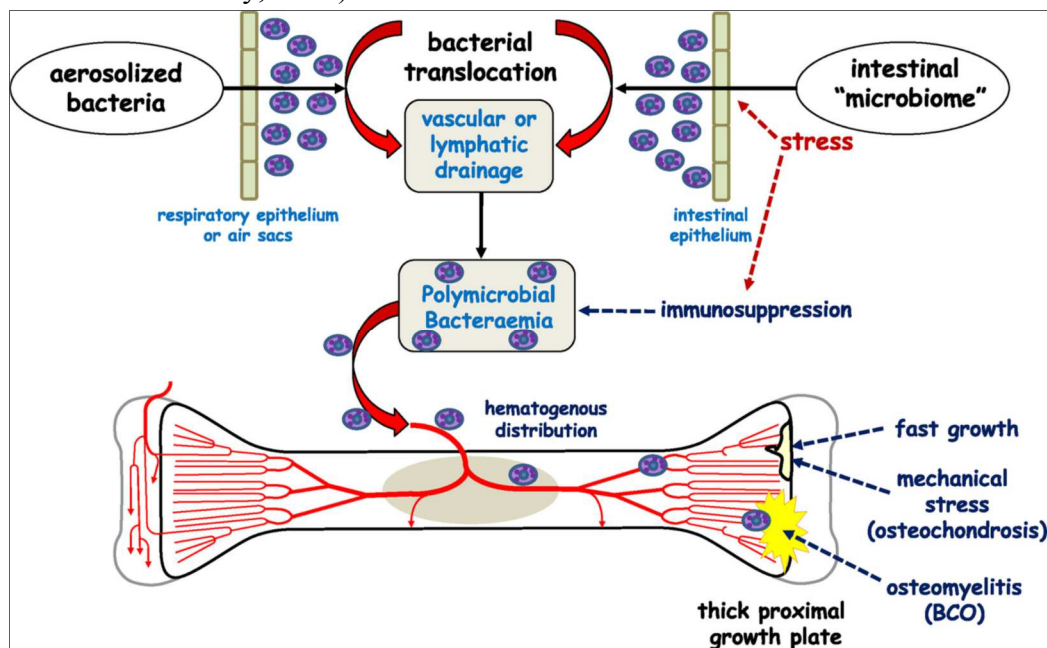




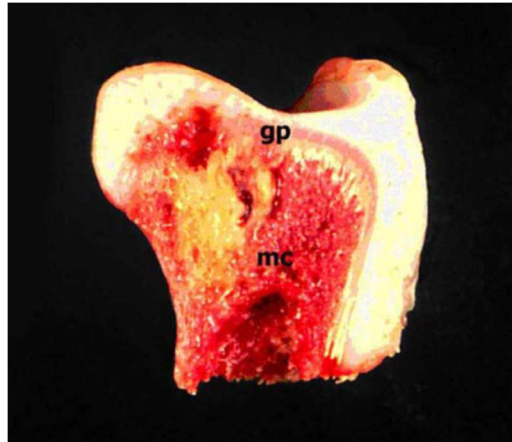
**Figure 1.6.** Genetic selection methods have resulted in a change in the size of commercial broiler chickens. Broilers from the size of the University of Alberta Meat Control strains unselected between 1957 (left) and 1978 (middle), relative to Ross 308 broilers (2005; right). These birds were bred in the same environment and were of the same ages, day 0, 28, and 56, while their weights were 905, 1808, and 4202 grams, respectively. The Figure 1.6 was copied from (Zuidhof et al., 2014).



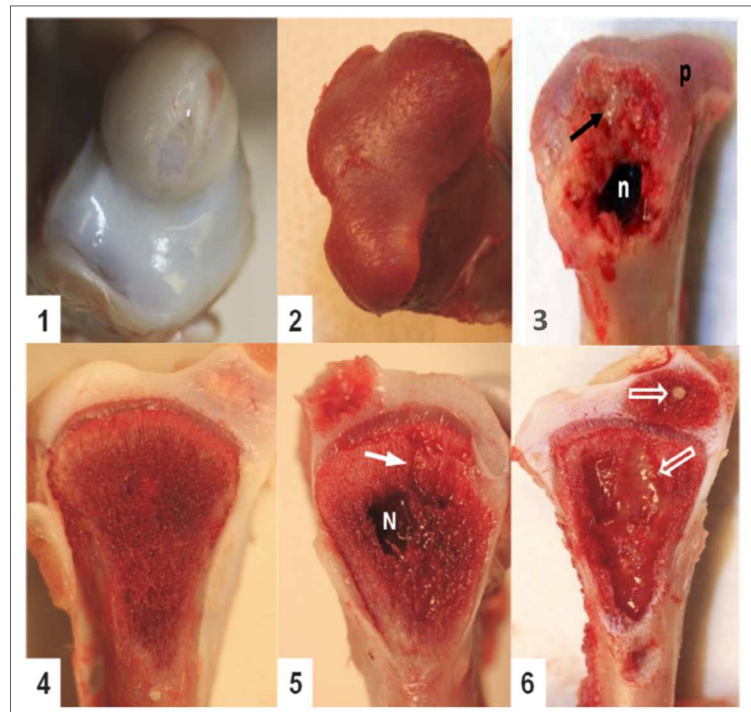
**Figure 1.7.** The illustration on the left pane and the photograph on the right pane illustrates the blood flow and anatomical form of the long bone in rising broilers (Copied from Wideman & Prisby, 2013).



**Figure 1.8a.** Pathways of bacterial infection in rapidly growing birds fast-growing growth plates and the neighboring metaphysis that contribute to the BCO pathogenesis. Bacteria from parent breeders, contaminated hatcheries, egg shells, or bacteria that enter the bird's circulatory system through the integument, respiratory system, or gastrointestinal tract are hematogenously distributed and colonize osteochondrotic crypts through microfractures triggered by a badly mineralized chondrocyte layer and caused by mechanical stress (This picture was copied from Wideman, 2016).

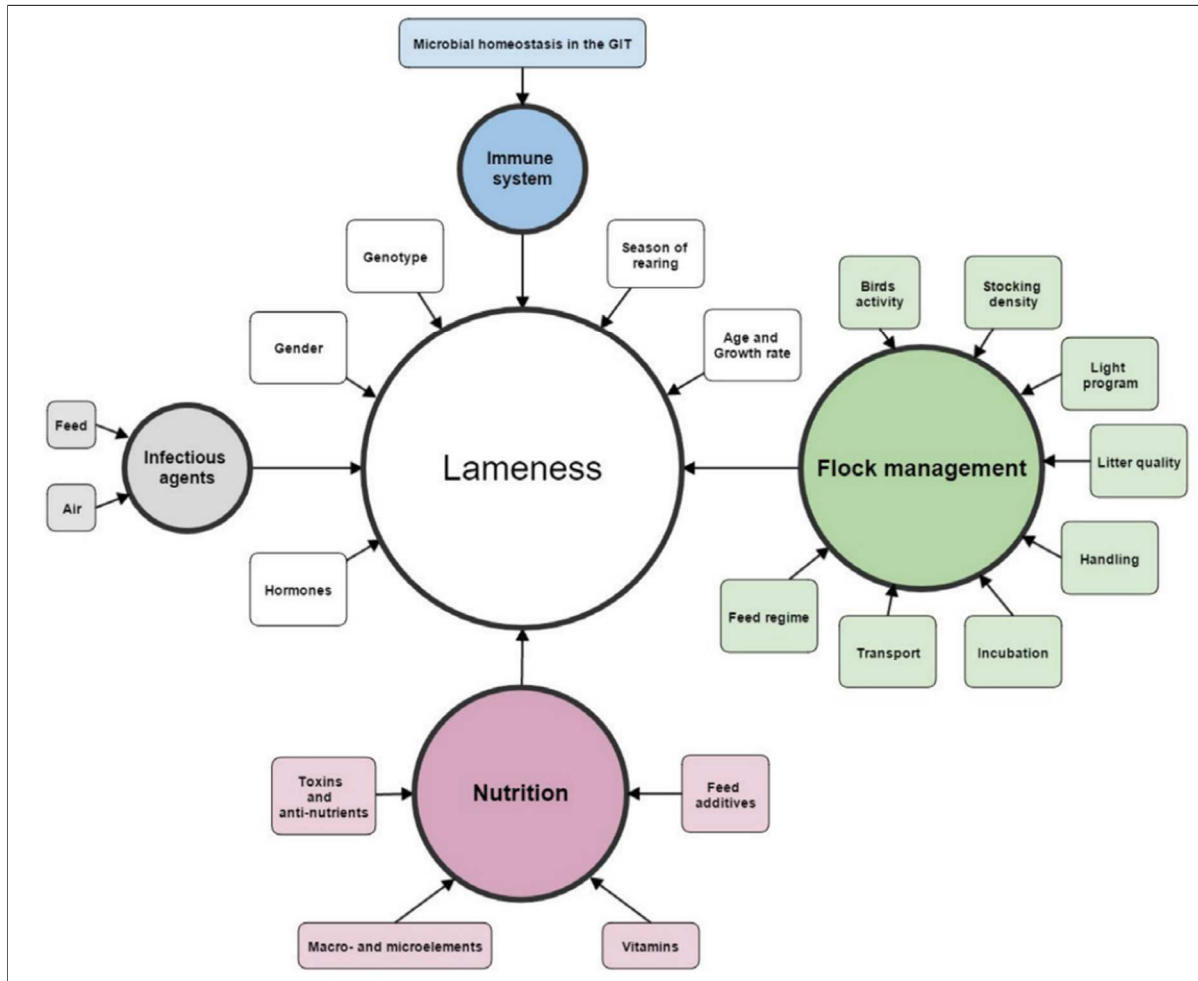


**Figure 1.8b:** The proximal end of the femur. The midline frontal segment of a 28-day-old lame commercial bird with BCO revealed a macroscopic yellow tissue lesion stretching from both the growth plate (gp) and the medullar cavity (mc). Photograph copied from (McNamee & Smyth, 2000).



**Figure 1.8c:** Stages of proximal femoral (upper row) or tibial (lower row) head degeneration progressing gradually to bacterial chondronecrosis with osteomyelitis (BCO): (1) Normal proximal femoral head; (2) Femoral head separation (FHS or epiphyseolysis); (3) Disarticulation of femur showing the underlying surface of growth plate or physis (p) with necrotic voids (n) and fibrinonecrotic exudate (black arrow) which display typical characteristics of femoral head necrosis (FHN) with osteomyelitis; (4) Normal proximal tibial head with spicules of trabecular bone in the metaphyseal zone which provide a supportive scaffold for the growth plate; (5) tibial head necrosis (THN) showing necrotic voids (n) in the metaphyseal zone which results in microfractures of the growth plate, lytic channel (white arrow) penetrate from necrotic voids into

the growth plate; (6) Tibial head necrosis severe or caseous (THNsc) showing regions of caseous bacterial infiltration and sequestrate (open arrows) which indicate macroscopic evidence of osteomyelitis. Photograph copied from (Jiang et al., 2015).



**Figure 1.9:** Contagious and non-contagious factors associated with the incidence of lameness in broilers (Image copied from Kierończyk *et al.*, 2017).

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## **CHAPTER 2**

**Whole genome comparisons of *Staphylococcus agnetis* isolates from cattle and chickens.**

## **Chapter 2: Whole genome comparisons of *Staphylococcus agnetis* isolates from cattle and chickens.**

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Running Head: *S. agnetis* phylogenomics

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### **Abstract.**

*S. agnetis* has been previously associated with subclinical or clinically mild cases of mastitis in dairy cattle and is one of several Staphylococcal species that have been isolated from the bone and blood of lame broilers. We reported that *S. agnetis* could be obtained frequently from bacterial chondronecrosis with osteomyelitis (BCO) lesions of lame broilers [Al-Rubaye *et al.*, PLoS One 10:e0143336, 2015, <https://doi.org/10.1371/journal.pone.0143336>]. A particular isolate, *S. agnetis* 908, can induce lameness in over 50% of exposed chickens, exceeding normal BCO incidences in broiler operations. We reported the assembly and annotation of the genome of isolate 908. To better understand the relationship between dairy cattle and broiler isolates, we assembled 11 additional genomes for *S. agnetis* isolates, an additional chicken BCO strain, and

ten isolates from cattle milk, mammary gland secretions or udder skin, from the collection at the University of Missouri. To trace phylogenetic relationships, we constructed phylogenetic trees based on multi-locus sequence typing, and Genome-to-Genome Distance Comparisons. Chicken isolate 908 clustered with two of the cattle isolates along with three isolates from chickens in Denmark and an isolate of *S. agnetis* we isolated from a BCO lesion on a commercial broiler farm in Arkansas. We used a number of BLAST tools to compare the chicken isolates to those from cattle and identified 98 coding sequences distinguishing isolate 908 from the cattle isolates. None of the identified genes explain the differences in host or tissue tropism. These analyses are critical to understanding how Staphylococci colonize and infect different hosts and potentially how they can transition to alternative niches (bone vs dermis).

## **Importance.**

*Staphylococcus agnetis* has been recently recognized as associated with disease in dairy cattle and meat type chickens. The infections appear to be limited in cattle and systemic in broilers. This report details the molecular relationships between cattle and chicken isolates in order to understand how this recently recognized species infects different hosts with different disease manifestations. The data show the chicken and cattle isolates are very closely related but the chicken isolates all cluster together suggesting a single jump from cattle to chickens.

## **Introduction.**

In the US, skeletal problems are estimated to cost the broiler industry more than 100 million dollars annually (1-5). Lameness is an important chicken industry issue affecting from 1-10% of a flock, and a wide array of bacterial genera have been isolated from chickens affected by bacterial chondronecrosis with osteomyelitis (BCO) (5-25). *Staphylococcus agnetis*, a coagulase-variable, Gram-positive bacterium has been found to cause infections of the bones and blood of broilers leading to BCO (26, 27). BCO primarily affects the growth plate in the proximal femur and tibia, the fast-growing leg bones. We have shown that an isolate of *S. agnetis* (strain 908) obtained from BCO chickens can induce BCO lameness at levels greater than 50% of the population when administered in a single dose in drinking water (26, 27). Previously, *S. agnetis* has also been associated with subclinical or mild cases of clinical mastitis in dairy cattle (28-31). There are very few reports of *S. agnetis* in poultry and we have speculated that the virulent strain we isolated may be the result of prolonged selection resulting from years of inducing BCO lameness at our research farm. Genome sequence analysis of multiple isolates of *S. agnetis* from the University of Arkansas research farm have revealed little sequence variation and thus they



appear to be clonal (unpublished data). The annotated complete genome of strain 908 has been published (26). Draft genomes of a cattle isolate, *S. agnetis* CBMRN20813338 (32), and chicken isolates (33) have been deposited in the NCBI genome databases. To better understand the phylogenomic relationships between dairy cattle and broiler isolates, we have generated genome assemblies for multiple cattle isolates and an additional chicken isolate of *S. agnetis*. We used multi-locus sequence typing (MLST) and genome distance comparisons to develop phylogenetic trees. We also performed reciprocal BLAST and BLASTX comparisons to identify genes and gene islands that distinguish the chicken and cattle isolates. The goal was to determine the phylogenetic relationships between cattle and chicken isolates, and whether there were easily discernable genes responsible for the virulence of isolate 908, or species-specific pathogenesis.

## **Results.**

### ***Staphylococcus agnetis* genomes assemblies.**

Sources (host, tissue, disease) for the *S. agnetis* isolates used in our analyses are presented in Table 2.1. For this work, we generated draft genomes for eight cattle isolates (1383, 1384, 1385, 1387, 1389, 1390, 1391, 1392) from 2x251 paired end MiSeq reads (Table S2.1), and we generated one finished genome for one cattle isolate (1379). These cattle isolates were cultured from skin swabs (1385, 1389), milk (1379, 1383, 1384, 1387, 1391, 1392), or pre-partum mammary gland secretions (1390). The new, draft, *de novo* assemblies for the eight cattle isolates ranged from 43 to 328 contigs comprising 2.381 to 2.581 Mbp (Table S2.1). The hybrid assembly from long and short reads (see Materials and Methods for details) for cattle isolate 1379 produced a single chromosome of 2.45 Mbp. We identified *S. agnetis* isolate 1416 from a BCO lesion in a necropsy sampling of BCO birds on a commercial broiler farm in Arkansas. The

hybrid assembly of the 1416 genome produced a 2.45 Mbp chromosome and plasmids of 59 and 28 kbp. We included the draft genome for cattle isolate CBMRN20813338 as it was the first *S. agnetis* genome characterized (32). We had earlier published the finished genome for chicken isolate *S. agnetis* 908, a *de novo* assembly of Pacific Biosciences long reads, with subsequent correction with MiSeq reads (26). This assembly includes a single 2.47 Mbp chromosome and a 29 kbp plasmid. Recently, we identified two additional plasmids of 3.0 and 2.2 kbp (unpublished) from the assembly data that we have included in our genome comparisons.

**Phylogenetic analyses.** To begin to trace the phylogenomic relationship between the cattle *S. agnetis* isolates and those from chicken, we first generated MLST phylogenetic trees. We included a total of 13 isolates, including the published genome for cattle isolate *S. agnetis* CBMRN20813338, the 9 new cattle isolate assemblies, chicken isolates 908 and 1416, and *S. agnetis* isolate 12B from NCBI. We included the genome from strain 12B, isolated from the milk of a buffalo with bubaline mastitis. The dendrogram from genome BLAST on the NCBI genome page for *S. agnetis*, presents 12B as the closest genome for a bovine isolate to the genome for chicken isolate 908. *S. hyicus* ATCC11249<sup>T</sup> (34) from swine exudative dermatitis was used as the outgroup. Figure 2.1 presents a tree based on seven housekeeping genes (*ackA*, *fdhD*, *fdhF*, *grol*, *purA*, *tpiA*, *tuf*), where orthologs could be identified in each of the assemblies, and the genes are dispersed throughout the 908 chromosome. From the MLST phylogenetic tree, we see two chicken isolates (908 and 1416) cluster within the cattle *S. agnetis* isolates within a clade with bovine isolates 1379, 1387 and 12B. MLST analysis with seven virulence genes (encoding five distinct fibronectin binding proteins and two exotoxins) identified in all of the assemblies produced a tree with a very similar topology (data not shown).

Since we began our analyses, additional genomes for isolates of *S. agnetis* have been deposited in NCBI. The NCBI dendrogram based on genomic BLASTN (<https://www.ncbi.nlm.nih.gov/genome/?term=agnetis>) for 26 *S. agnetis* assemblies, indicates that our 908 chicken isolate clusters with three Danish chicken isolates and 5 bovine isolates (12B, SUNC\_2265, SNUC\_4805, SNUC\_5151, SUC\_3261) in a single clade relative to CBMRN20813338, and 16 additional bovine isolates. In order to expand on the MLST analyses and all 36 genomes (26 in NCBI and our 10 new assemblies, including 9 isolates of bovine origin and 1 isolate of chicken origin) we used the Genome-to-Genome Distance Calculator (GGDC) to generate a phylogenetic tree based on genetic distances computed from whole genome BLASTN comparisons (35). This included our two chicken isolates (908, 1416) and three chicken isolates (NEDS, NEFX, NDYM) from organs from two deceased broilers on a farm in Denmark (33). The phylogenetic tree based on genome distances (Fig. 2.2) shows that four of the genomes from chicken isolates (908, NEDS, NEFX, NDYM) cluster within the cattle isolates with the Denmark chicken isolates being most similar to our chicken isolate 908. Chicken isolate 1416 is in a sister branch clustered with 7 bovine isolates including isolate 12B from the milk of a buffalo in Argentina with mastitis. The data are consistent with five, or potentially six, different clades within the *S. agnetis* species group with the five chicken isolate genomes all within one clade. The nine new genomes for mastitis-related isolates of *S. agnetis* from the USA are distributed across all branches of the tree. There is no indication of geographic restriction of particular genotypes for *S. agnetis* isolated from the bovine mammary gland. Nor is there a particularly noticeable separation of the chicken isolate genomes from the cattle isolate genomes. We also analyzed all of the genomes by Average Nucleotide Identity (36) and obtained the same phylogenomic architecture (data not shown).

**Genome Comparison.** We used CGView Server (37) to perform and visualize comparisons of the 2.47 Mbp chromosome from chicken isolate 908 to three cattle isolate genomes; the finished 1379 isolate genome and draft genomes for isolates 1387 and 1385 (Fig. 2.3). We selected the 1379 isolate genome for production of a finished genome based on being one of the closest genomes to the chicken isolates (Fig. 2.2). We included the draft 1387 isolate genome from the same branch as the chicken isolates, and 1385 as the largest assembly of the other cattle isolates. The CGView in Figure 2.3 identified five gene islands which appear to distinguish chicken isolate 908, from the three cattle isolates. The five islands were also visible when we compared chicken isolate 908 to our other new draft cattle assemblies or the buffalo isolate 12B (data not shown).

We hypothesized that these islands could potentially contain sequences related to host adaptation. We annotated the genes in these five islands using BLASTP and further evaluated for presence in the other four chicken isolate genomes, or any of the currently available 36 bovine isolate genomes (Table S2.2). Regions in the 908 genome not represented in the cattle isolates according to the CGView are located approximately as follows: island 1 for 167-235 kbp; island 2 for 978-1021 kbp; island 3 for 1162-1177 kbp ; island 4 for 1831-1848 kbp; and island 5 for 2007-2018 kbp. Thus, approximately 154 kbp out of 2474 kbp are distinct from the cattle isolates.

Analysis of the 908 2.47 Mbp chromosome for prophage using the PHASTER website (data not shown) identifies island 1 as containing two intact Staphylococcal prophage (Staphy\_EW\_NC\_007056 and Staphy\_IME\_SA4\_NC\_029025) from 170.1 to 232.7 kbp. Islands 2 and 3 are identified as questionable for being complete prophage. Island 2 is Staphy\_2638A\_NC\_007051 from 980.8 to 1020.9 kbp and island 3 is most similar to a

*Clostridium* phage, phiMMP04\_NC\_019422, from 1156.8 to 1178.5 kbp. Island 4 contains genes indicative of a conjugative transposon, with sequence similarity to Tn6012 of *S. aureus*, inserted in an intergenic region approximately 170 bp upstream of the *bioD* gene. The shortest of the five blocks is island 5 (~11 kbp), which contains an apparent operon encoding a strain variable Type 1 DNA restriction-modification system (*hsdMSR*). Therefore, 124.4 of the estimated 154 kbp in the five islands represents prophage sequences, while the other two contain a probable transposon and a restriction-modification operon. The most similar match to island 4 and 5 in BLAST searches at NCBI were to *S. aureus* genomes. Figure 2.4 further relates these 5 islands as candidates for host adaptation by comparing the 908 isolate 2.47 Mbp chromosome to the finished 1379 bovine isolate genome and draft assemblies of chicken isolates NEDS from the a deceased broiler in Denmark and the finished genome assembly of isolate 1416 from a BCO broiler on a commercial farm in Arkansas. From these comparisons we conclude that islands 1, 2, 4, and 5 are, for the most part, present in at least one of the other two chicken isolates, but none of the islands is in both of the other chicken isolates (i.e., specific to all chicken isolates). There is the caveat that the 908 isolate and 1416 isolate genomes are finished genomes and the NEDS genome is only a draft genome so any island not found in the NEDS assembly could potentially be an assembly issue. Close inspection of the TBLASTN analysis of the proteins from the islands for presence in any of the bovine isolate genomes (Table S2.2) 164 of the 908 predicted polypeptides have significant matches in at least one of the cattle isolates, while only 32 are not found in any of the cattle isolates. For those 32 polypeptides, 31 are also not found in either of the four other chicken isolates (1416 or the three Danish isolates). Polypeptide 217 is the only polypeptide not identified in any bovine isolate genome but is identified in the 1416 genome. Polypeptide 217 is a 47 amino acid hypothetical protein, so we see no real islands of

polypeptides (i.e., chicken specific pathogenicity island) that distinguish chicken isolate genomes from the bovine isolate genomes.

We have assembled sequences of three plasmids in isolate 908 (29, 3 and 2.2 kbp). None of these plasmids is found in any of our nine newly assembled cattle isolate listed in Table 2.1. There are presently 26 genome assemblies for *S. agnetis* in the NCBI database. Only two assemblies are listed as “completed” (i.e., finished), our assembly for chicken isolate 908 (26), and isolate 12B from buffalo milk in Argentina (unpublished). The other 24 are draft assemblies. The NCBI genomes include 21 isolates from cattle, one from buffalo, and four chicken isolates; 908 and the three chicken isolates from Denmark (NEDS, NEFX, and NDYM). We performed a BLASTN search using the NCBI program selection “optimize for highly similar sequences (megablast)” where the query was the three plasmids from isolate 908 plasmids and the database searched was the 26 assemblies already in NCBI. The 29 kbp plasmid identified one 4729 base contig in the NEFX assembly with 22% query coverage in 10 different regions, with the largest region comprising 3317 identities over 3331 bases. The 3 kbp plasmid matched 450 out of 478 bases in 565 bp contigs in all three of the Danish chicken isolate assemblies (NDYM, NEDS, NEFX). The 2.2 kbp plasmid matched 2071 out of 2080 bases in a 2304 base contig in the chicken NEFX assembly. We also performed BLASTN searches of the three plasmids using the NCBI NR database exclusive of *S. agnetis* entries. The best matches for the 29 kbp plasmid are to the 30.9 kbp plasmid pH1-1 from a pheasant isolate of *S. aureus*. The two plasmids share 99% identity with 39% query coverage in three different regions of the plasmid (2520, 1573, and 982 bp). The 3 kbp 908 plasmid has a 43% query cover with 89% identity with an unnamed 37.2 kbp *S. aureus* plasmid from a human isolate of *S. aureus*. The best match for the 2.2 kbp 908 plasmid was 99% identity for 2076 bp in a 46.5 kbp plasmid pSALNT46 from an *S. aureus*

isolate from retail turkey meat. We therefore conclude that none of the plasmids in isolate 908 appear to correspond to a chicken host specialization determinant for the jump from to chickens, but some sequences in the 29 kbp plasmid and the 2.2 kbp plasmid could have been picked up after the jump to poultry.

To screen at higher resolution, we used the SEED Viewer sequence comparison tool to compare entire assemblies for individual polypeptide coding sequences for four isolates: chicken isolate 908 (including the 3 plasmids), chicken isolate 1416, bovine isolate 1379 and the bovine CBMRN isolate. We used isolates 908 and 1416 as finished assemblies of two chicken isolates, 1379 as a finished assembly of a cattle isolate, and CBMRN as the original draft cattle isolate. The BLASTP comparison results were then filtered for isolate 908 polypeptides with >90% identity for polypeptides in isolate 1416, but <50% identity for isolate 1379 and the CBMRN isolate. This filter identified 99 polypeptides (Table S2.3). To predict functions of these 99 polypeptides we used both the RAST annotation and the NCBI Prokaryote Genome Annotation Pipeline (PGAP) to categorize the potential function of these 99 polypeptides. We identified 75 polypeptides as hypothetical or of unknown function, 11 phage related, and 8 involved in mobile elements or plasmid maintenance. The remaining five polypeptides, listed under “other”, are: deoxyuridine 5'-triphosphate nucleotidohydrolase (EC 3.6.1.23), hypothetical SAR0365 homolog in superantigen-encoding pathogenicity islands SaPI, ribosyl nicotinamide transporter PnuC-like, aspartate aminotransferase (EC 2.6.1.1), and N-acetyl-L,L-diaminopimelate aminotransferase (EC 2.6.1.-). If we relaxed the cutoff for the cattle isolates to <70% identity in cattle isolates we identified 9 additional polypeptides which added one additional hypothetical polypeptide, four additional phage related polypeptides, and four additional polypeptides involved in mobile

element or plasmid maintenance. There were no additional polypeptides in the “other” category, only the five described above (Table 2.S3).

The dUTP nucleotidohydrolase (Gene ID 209; 191,107-191,625 bp) is annotated as a phage related protein with roles in viral replication for reducing incorporation of uracil in viral DNA and is located within the Staphy\_EW\_NC\_007056 prophage in island 1 described above, so this gene is likely to function primarily in the biology of that prophage.

The SAR0365 homolog (Gene ID 1037; 1,018,987-1,020,928 bp) is encoded in island 2 within the Staphy\_2638A\_NC\_007051 prophage. SAR0365 polypeptide is a hypothetical protein that the NCBI Prokaryotic Genome Annotation Pipeline annotates as a toxin in the PemK/MazF type II toxin-antitoxin system. Four of the seven protein entries for SAR0365 homologs in NCBI are associated with superantigen-encoding pathogenicity islands (SaPI) in clinical isolates of *Staphylococcus aureus* and two are associated with *S. aureus* phages. Mobilization of SaPI has been associated with temperate phage replication (38). The 908 isolate genome contains additional hypothetical genes annotated as SaPI-associated homologs (Gene ID 1184 1,173,209-1,173,412 bp; Gene ID 1185 1,173,409-1,173,714 bp; Gene ID 1938 1,957,610-1,959,703 bp; Gene ID 1939 1,959,935-1,961,452 bp; Gene ID 2116 2,136,487-2,136,603 bp). Mobilization depends on a terminase (38), but the only SaPI associated terminase is Gene ID 2092 (2,118,053-2,118,355 bp). We had previously described a cluster of five exotoxin/superantigen-like proteins from 1,956,884 to 1,968,958 bp (26). Therefore, the only potential superantigen-containing pathogenicity island would approximate from 1.95 to 2.12 Mbp which would be larger than the prototypical 15-18 kbp SaPI (38). A BLASTP of the *S. agnetis* protein database at NCBI with the predicted protein for Gene ID 1037 (SAR0365 homolog) identified the isolate 908 entry, as well as identical entries in all three Danish chicken



isolates (NEDS, NDYM, NEFX), but no entries in any of the 20 cattle *S. agnetis* isolates in NCBI. Expanding the BLASTP to all Staphylococcaceae identified highly similar matches (92% identity, 100% query coverage) in *Staphylococcus hominis* and less similar (50% identity, 98% query coverage) in *S. aureus*. Further analyses and additional samples would be required to speculate further regarding the role of this SAR0365 homolog as a virulence factor in chicken tropism.

The genes for ribosyl nicotinamide transporter (Gene ID 2466), aspartate aminotransferase (Gene ID 2469) and N-acetyl-L,L-diaminopimelate aminotransferase (Gene ID 2470) are located in a five gene region on the 29 kbp plasmid, with the other 2 genes encoding hypothetical polypeptides. We performed a BLASTP search of the Staphylococcaceae proteins in the NCBI database. The 89 amino acid ribosyl nicotinamide transporter matched multiple entries from *S. aureus* and all three Danish *S. agnetis* isolates from chicken. Many of the BLASTP hits for this polypeptide are annotated as an AbrB family transcriptional regulator by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The 64 amino acid hypothetical polypeptide for Gene ID 2467 is only conserved in two entries from the Danish *S. agnetis* broiler isolates. The 197 residue polypeptide from Gene ID 2468 is well conserved in a broad swath of staphylococci and PGAP annotates this polypeptide as an IS6 family transposase. We note that Gene ID 2469 and 2470 are close to each other and in different reading frames suggestive of a possible frameshift introduced as an assembly error. Indeed, if we join the predicted polypeptides of these two ORFs, BLASTP analysis identifies *Staphylooccus* protein entries that match over the span of the merged polypeptides. However, we have evaluated this hypothesis further by templated assembly of the 908 MiSeq data onto the 29 kbp plasmid sequence. The MiSeq reads all agree with the assembly as presented in our NCBI submission. Therefore, these

two ORFs in the 29 kbp plasmid may be a frameshifted pseudogene or, if translated may have alternate functions for this organism.

Finally, to determine whether there were any regions in the cattle isolates that are not found in the chicken isolate genome assemblies, we performed a CGView analysis with the finished genome from isolate 1379 as the reference (Fig. 2.5). We included cattle isolate CBMRN and compared to the finished genomes of chicken isolates 908 (chromosome plus three plasmids), and 1416. There were cattle isolate regions that appeared to be absent from one of the chicken isolates but there were no regions found in both cattle isolates that were missing from both chicken isolates.

## **Discussion.**

The *Staphylococcus* genus not only includes a number of pathogenic species infecting vertebrate animals worldwide, but also includes many saprophytic or commensal species (39-41). *S. agnetis* is closely related to *S. hyicus* and *S. chromogenes* and was only described as a distinct staphylococcal species in 2012, based on DNA sequence differences of rDNA and two protein coding genes in isolates from mastitis in dairy cattle (42). *S. agnetis* cannot be easily differentiated from *S. hyicus* using routine speciation techniques, e.g. partial 16S rDNA sequencing, MALDI-TOF, or fermentation methods (28-30, 43). Hence, *S. agnetis* has either escaped recognition due to misclassification or is an emerging pathogen in some agricultural animal species. While *S. agnetis* was originally reported in cattle mastitis (42), it has more recently been reported in chicken bone infections and in multiple internal organs from deceased broilers (33). Metagenomics also detected *S. agnetis* 16S rDNA sequences in the gut of a sheep scab mite (44). Phylogenetic analyses based on 16S rRNA sequences cluster *S. agnetis* very

close to *S. hyicus* (26) with a group of staphylococci associated with domestic vertebrate species (e.g., cattle, swine, dog) (28-31, 34, 45-53). Most of these species are associated with dermal or epithelial infections, such as exudative dermatitis (28, 29, 31, 34, 47, 49-51, 53-55) and not with osteomyelitis as we have seen with chicken isolate 908 (26, 27). The more phylogenetically distant taxon, *S. aureus*, is prominently known for osteomyelitis in humans (56-58). The Danish broiler chicken isolates were from multiple tissues from deceased birds and we have no information about possible osteomyelitis. That the three Danish *S. agnetis* isolates, and our isolates 908 and 1416, are all closely related and within a clade of the cattle *S. agnetis* isolates suggests a recent expansion of the host range (i.e. from cattle to chickens) as seen for a human-specific clade of *S. aureus* that “jumped” to chickens in the United Kingdom (17). A single radiation out of the cattle group also argues against *S. agnetis* jumping back and forth between cattle and chickens. We have previously reported that isolate 908 can produce a bacteremia in the latter stages of BCO development before the birds are overtly lame (5, 26, 27, 59). We do not know if the Danish isolates can induce the BCO lameness that we have demonstrated for isolate 908 (26, 27). Our isolate 908 appears to represent a hypervirulent clone expanded through years of inducing high levels of BCO lameness on our research farm (5, 26, 27, 60-63) and could have evolved through selection from less virulent *S. agnetis* in broiler populations. Therefore, our genomic comparisons have been directed towards identification of any gene(s) that *S. agnetis* 908 could have acquired that facilitate the switch from involvement in cattle epithelial and mammary gland colonization and infection, to bone infections in chickens.

None of the gene islands (Table S2.2) or individual genes (Table S2.3) we identified as distinguishing isolate 908 from closely related cattle isolates is currently recognizable as a virulence marker, or that mediates tissue tropism. Previously we had identified 44 virulence

genes in our annotation of the isolate 908 genome (26) and none of these genes is in the regions distinguishing the chicken and cattle isolates. The genomic analyses of the human-to-chicken jump for *S. aureus* was associated with acquisition of two prophage, two plasmids, and a pathogenicity island, the inactivation of several virulence determinants important to human pathogenesis, and enhanced resistance to chicken neutrophils (17). Thus, we expected to readily find genes, or gene clusters, in chicken isolates of *S. agnetis* associated with the jump to chickens from cattle. Most of the distinguishing gene islands in isolate 908 contain genes associated with mobile elements (prophage), but none are known virulence determinants. We have unpublished evidence that isolate 908 is highly resistant to an immortalized chicken macrophage and are pursuing the genes for macrophage resistance. Since we have failed to identify unique virulence genes that distinguish the chicken isolate 908 from the cattle isolates we conclude that the basis for the jump from cattle to chickens is most likely the result of small alterations (i.e., missense or regulatory mutations ) in a few virulence-associated factors. Hypervirulence of isolate 908 in chickens could be from a single amino acid change. Hypervirulence of isolates of *Campylobacter jejuni*, were demonstrated to result from a single substitution in an outer membrane protein, resulting in induction of spontaneous abortions in sheep (64). Therefore, further fine-level comparisons or directed genome evolution (64) will be required to dissect how this emerging pathogen has evolved and diversified from cattle mastitis to chicken bone pathogen.

## **Materials and Methods**

**Reference genomes.** Isolate designations and host sources are provided in Table 2.1 and the details of the genome assemblies and accessions in NCBI are in Table 2.S1. Chicken isolate 908 was from necrotic femoral lesions, while NDYM, NEDS and NEFX were from tissue samples

from deceased broilers in Denmark. Isolates NDYM and NEDS were from the same broiler. The cattle isolate CBMRN was isolated from milk of a cow with subclinical mastitis that was enrolled in the Canadian Bovine Mastitis Research Network (CBMRN) cohort study (32). ATCC11249 represents the *S. hyicus* type strain isolated from a pig exudative epidermitis lesion (34).

**Bacterial strains.** Genomes for ten *S. agnetis* isolates were newly assembled (Table S2.1), including nine cattle isolates, and one chicken isolate. Chicken isolate 1416 was isolated from a necrotic femoral lesion of a lame bird in a commercial broiler operation in Arkansas. The nine cattle isolates were from a collection at the University of Missouri. Two isolates were skin isolates, one isolate was obtained from a pre-partum mammary secretion, and six isolates were obtained from the milk of cows with subclinical mastitis. All cattle isolates had been previously identified as *S. agnetis* based on partial DNA sequence of either elongation factor Tu (*tuf*) or 3-dehydroquinate dehydratase (*aroD*) (30). All isolates were archived at -80 °C in 20-40% glycerol, maintained on Tryptic Soy Agar slants, and grown in Tryptic Soy Broth (TSB; Difco, Becton, Dickinson and Company, Franklin Lakes, NJ).

**Genome sequencing and assembly.** DNA isolation was based on the method described by Dyer and Iandolo (65). Isolates were grown to mid log phase in TSB (40 ml) at 37 °C with shaking, pelleted, and resuspended in 2.5 ml 30 mM TrisCl, 3 mM EDTA, 50 mM NaCl, 50 mM glucose, pH 7.5. Lysostaphin (Sigma-Aldrich, St. Louis, MO) was added to 20 µg/ml, and incubated at 37 °C for 40-60 min. SDS was added to 0.5%, then the lysate was treated with RNaseA (Sigma-Aldrich) at 20 ug/ul for 30 min at 37 °C, then Pronase E (Sigma-Aldrich) at 20 ug/ul for 30 min

at 37 °C. The lysate was then extracted successively with 50:48:2 phenol:CHCl<sub>3</sub>:isoamyl alcohol, and 24:1 CHCl<sub>3</sub>:isoamyl alcohol. DNA was then collected by ethanol precipitation. DNA was quantified by Hoechst 33258 fluorometry in a GloMax®-Multi Jr. (Promega Corp., Madison, WI), and DNA integrity verified by agarose gel electrophoresis. Purified DNA from each isolate was submitted to the Research Technology Support Facility Genomics Core at Michigan State University for barcoded-library construction, pooled and subjected to 2x251 sequencing on an Illumina MiSeq. For draft genome assemblies the MiSeq reads were assembled using the *de novo* pipeline in Lasergene NGen ver. 13.0 (DNASTar, Madison, WI). For isolates 1379 and 1416 we produced finished genomes by hybrid assemblies of MiSeq and Oxford Nanopore MinION long reads. Long reads were either from barcoded or rapid kit libraries prepared and sequenced on Minion v9.3 flow cells (Oxford Nanopore Technologies, Oxford Science Park, UK) according to the manufacturer's recommendations. Minion reads were filtered with a custom script to filter reads for length and average Q-score, prior to assembly. For isolate 1379, we filtered for length  $\geq 2000$  bases and Qscore  $\geq 13$ . For isolate 1416, we filtered for length  $\geq 5000$  bases and Qscore  $> 16$ . Nanopore reads and MiSeq reads were assembled using the Unicycler ver. 0.4.6.0 pipeline on Galaxy (<https://usegalaxy.org>) using the Bold bridging mode. All assemblies and sequence reads have been deposited in NCBI and the accession and biosample identifiers are in Table 2.S1.

**Genome annotation and phylogenetic comparison.** The assembled genome sequences were compared with chicken isolate 908 using BLASTN implemented in CGViewer (37) to identify regions missing in one or more genome. Specific gene regions were annotated using either the BASys server at <http://www.basys.ca> (66) or the Rapid Annotation using System Technology

(RAST) server at <http://rast.nmpdr.org> (67). Unique genes were verified by TBLASTN comparisons and reciprocal gene-by-gene BLASTP comparisons using the SEED server (68). Unique genes were further annotated using the KEGG (Kyoto Encyclopedia of Genes and Genomes) website at <https://www.genome.jp/kegg> (69). Prophage identification was performed using PHASTER (PHAge Search Tool Enhanced Release) at <http://phaster.ca> (70).

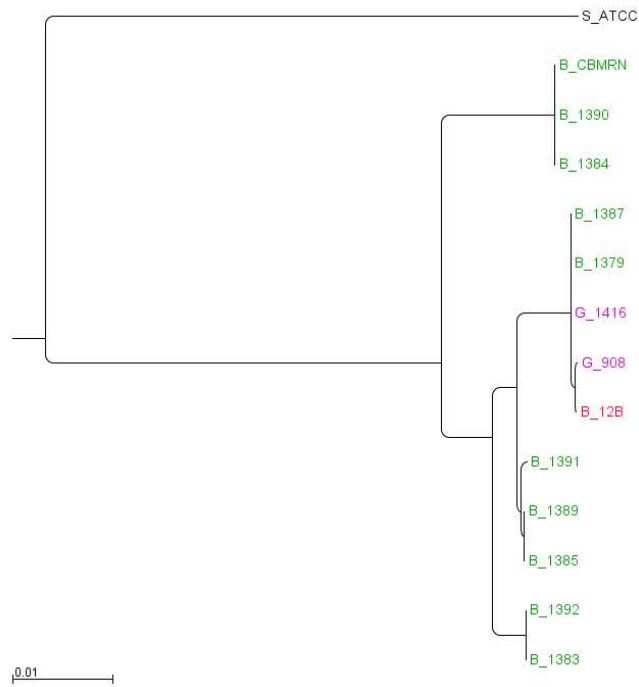
**Phylogenetic Analyses.** For MLST analysis gene coding sequences were aligned and trimmed in MegAlign (DNASar) then concatenated. Clustal Omega implemented in MegAlignPro (DNASar) was used to generate phylogenetic trees. Consensus neighbor-joining trees with 2500 bootstrap replications were constructed based on the alignments. Genome-to-Genome Distance Calculator (GGDC) <http://ggdc.dsmz.de/ggdc.php> (35) was used to generate whole genome BLAST distance values. These distance calculations were used to generate a phylogenetic tree using the neighbor-joining method as implemented at <http://trex.uqam.ca>. Trees were rendered and rerooted in Archeopteryx 0.9901 (71).

**Table 2.1.** Bacterial genomes utilized in these analyses. Genomes are separated by species designation and host source. For each genome the isolate designation is given as well as our abbreviation for this manuscript where indicated. The Isolate Source indicates tissue or sample source for the bacterial isolate. Genome status indicates whether the genome is considered finished or draft. Citation is the publication source. Further information on the genome assemblies is provided in Table 2.S1.

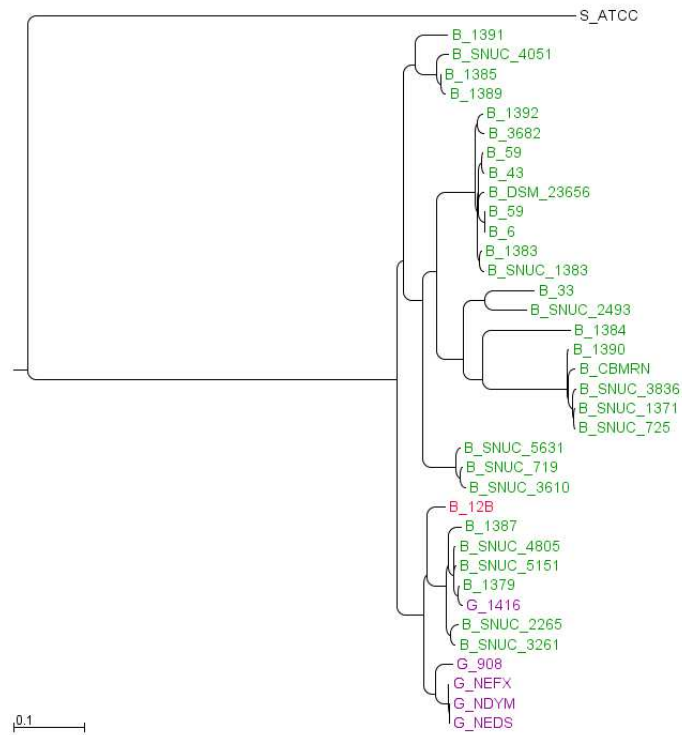
<b>Strain Designation (Abbreviation)</b>	<b>Isolate Source</b>	<b>Genome Status</b>	<b>Citation</b>
<i>S. hyicus</i> isolate from swine			
ATCC 11249 (ATCC)	swine exudative epidermitis	Finished	(34)
<i>S. agnetis</i> isolates from chickens			
1416	broiler femoral BCO lesion; Arkansas broiler commercial farm	Finished	This work (33)
722_230714_2_5_spleen (NEDS)	broiler spleen, deceased; Denmark	Draft	
722_260714_1_8_heart (NDYM)	broiler heart, deceased; Denmark	Draft	
723_310714_2_2_spleen (NEFX)	broiler spleen, deceased; Denmark	Draft	
908	broiler femoral BCO lesion; UA Research farm	Finished	(26)
<i>S. agnetis</i> isolates from bovine			
12B	buffalo milk		none
1379	bovine mammary gland - milk	Finished	This work
1383	bovine mammary gland - milk	Draft	
1384	bovine mammary gland - milk	Draft	
1385	bovine teat skin	Draft	
1387	bovine mammary gland – milk	Draft	
1389	bovine inguinal skin	Draft	
1390	bovine mammary gland – pre-partum mammary gland secretion	Draft	
1391	bovine mammary gland – milk	Draft	
1392	bovine mammary gland - milk	Draft	none
33	bovine	Draft	
3682	bovine milk	Draft	
43	bovine	Draft	
59 (59a)	bovine	Draft	
59 (59b)	bovine	Draft	
6	bovine	Draft	none
CBMRN 20813338 (CBMRN)	bovine mammary gland - milk	Draft	(32)
DSM_23656	bovine mastitic milk	Draft	none



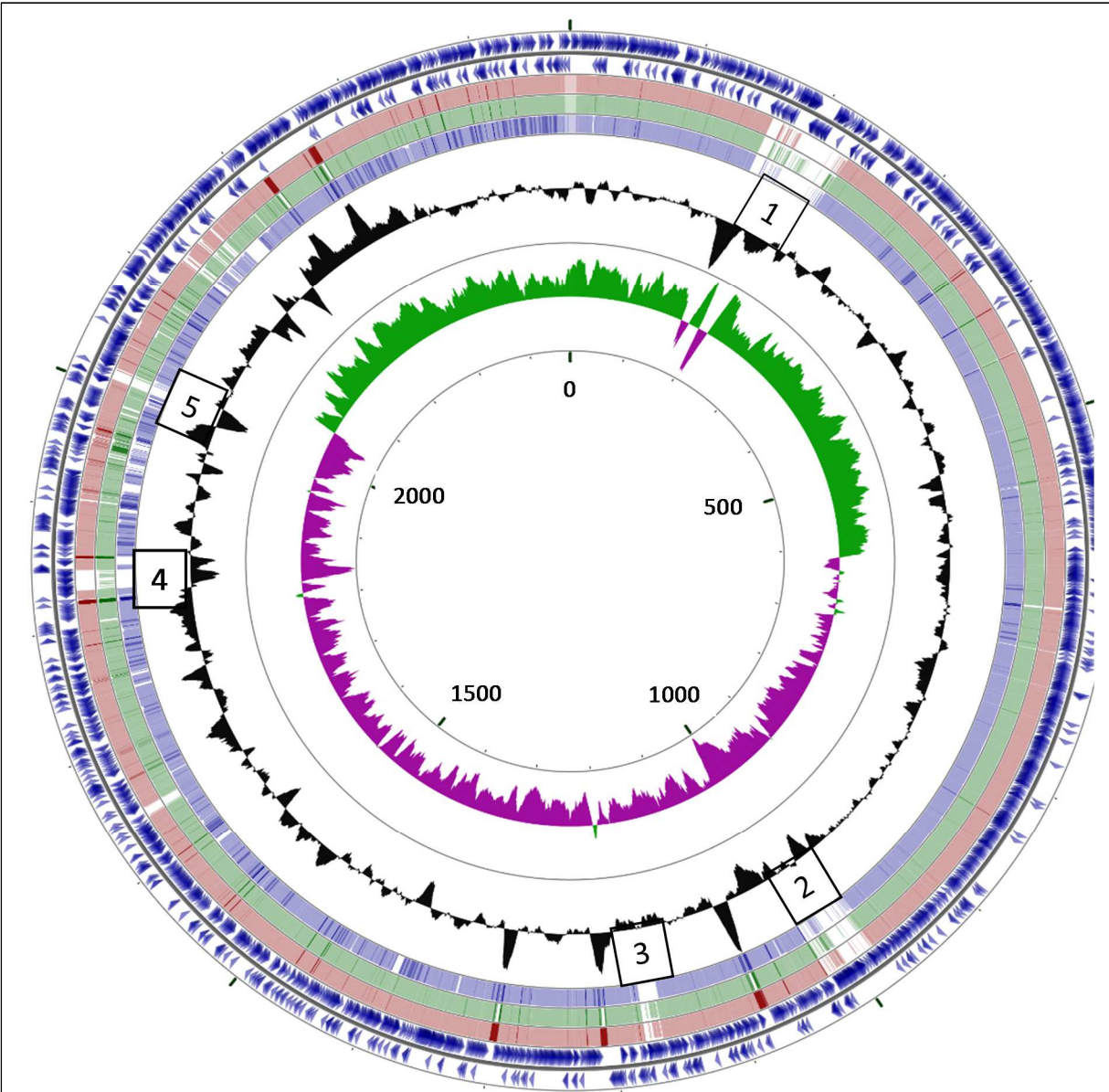
Strain Designation (Abbreviation)	Isolate Source	Genome Status	Citation
SNUC_1371	Holstein clinical mastitis	Draft	(72)
SNUC_1383	Holstein clinical mastitis	Draft	
SNUC_2265	Holstein subclinical mastitis	Draft	
SNUC_2493	Holstein subclinical mastitis	Draft	
SNUC_3261	Holstein subclinical mastitis	Draft	
SNUC_3610	Holstein subclinical mastitis	Draft	
SNUC_3836	Holstein subclinical mastitis	Draft	
SNUC_4051	Holstein subclinical mastitis	Draft	
SNUC_4805	Holstein subclinical mastitis	Draft	
SNUC_5151	Holstein subclinical mastitis	Draft	
SNUC_5631	Holstein subclinical mastitis	Draft	
SNUC_719	Holstein subclinical mastitis	Draft	
SNUC_725	Holstein subclinical mastitis	Draft	



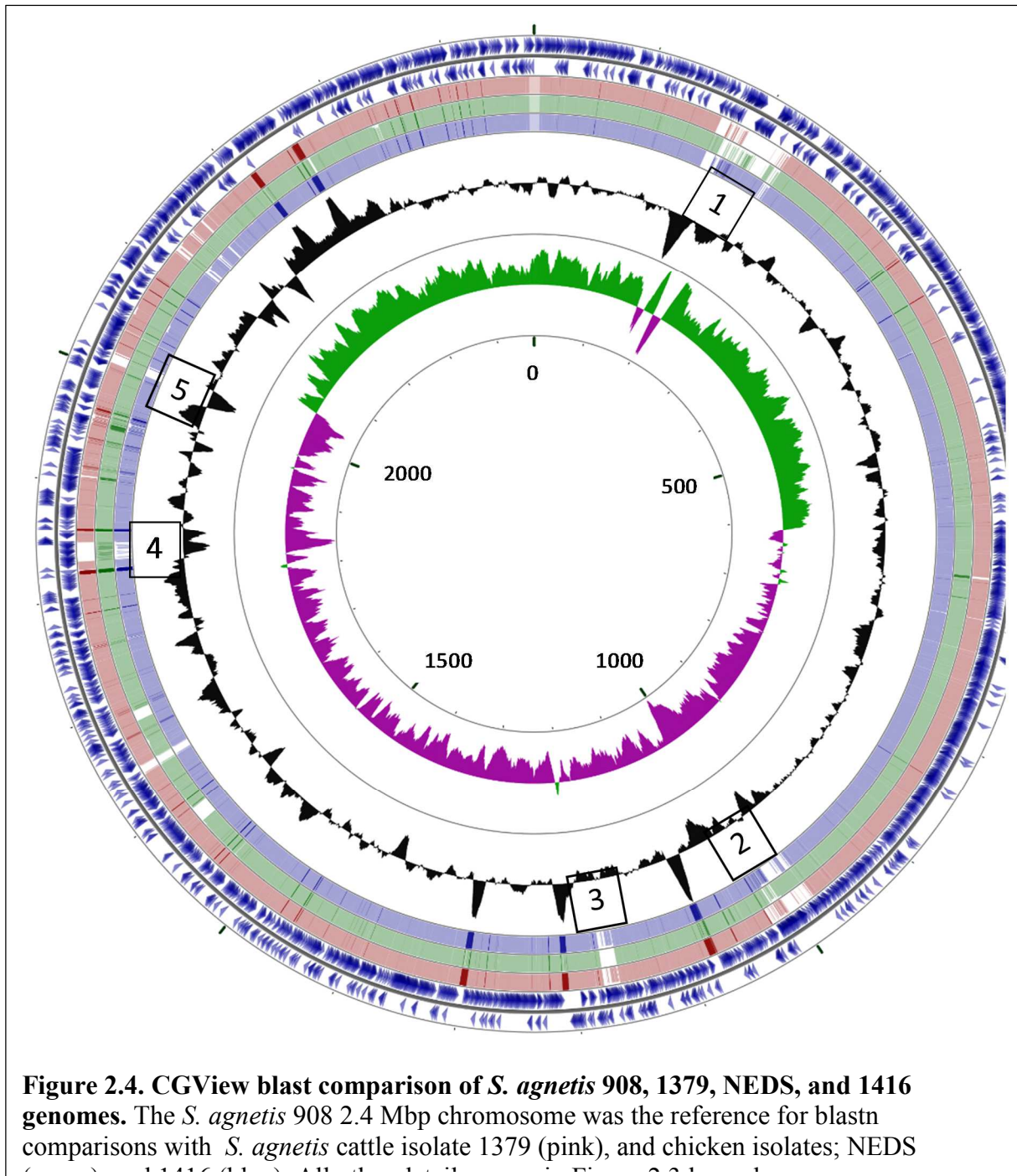
**Figure 2.1.** MLST phylogeny for two chicken and eleven bovine, isolates of *S. agnetis*, with a swine *S. hyicus* (ATCC) isolate as the out-group. The tree is based on seven housekeeping genes (see text). Isolates are coded for host source by prefix and color: chicken G\_ purple, cattle B\_ green, bison B\_ red, and swine S\_ black.



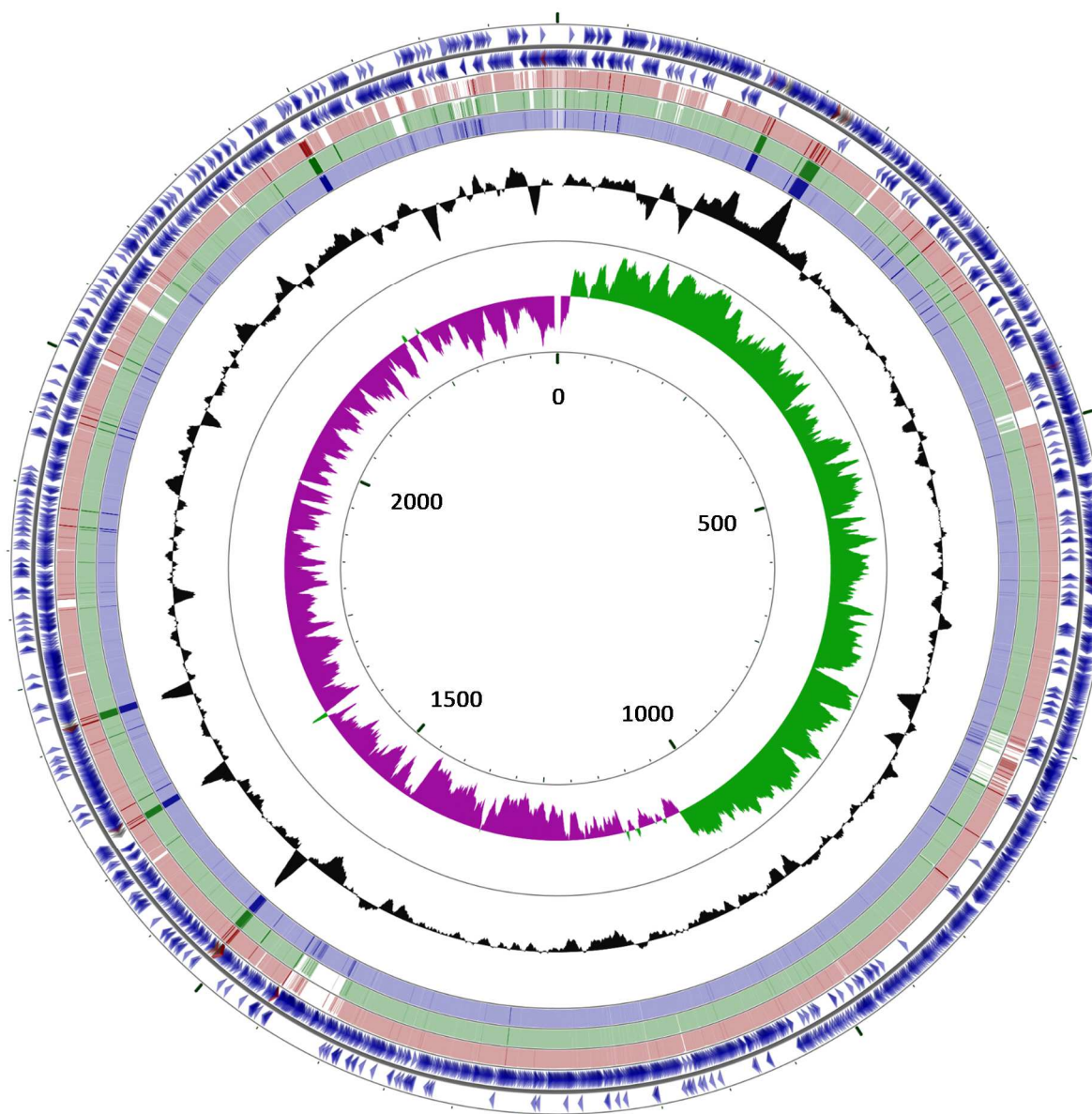
**Figure 2.2.** Genome to Genome Distance phylogenetic tree for 36 *S. agnetis* genomes. GGDC data (formula 2) was used to construct a neighbor-joining tree for comparison of five chicken, and 31 bovine isolates, of *S. agnetis*. *S. hyicus* (ATCC) from swine was the out-group. Isolate prefix and color coding as in Figure 2.1. Isolates are described in Table1, and genomes in Table 2.S1.



**Figure 2.3. CGView blastn comparison of *S. agnetis* 908, 1379, 1387 and 1385 genomes.** The *S. agnetis* 908 2.4 Mbp chromosome was the reference for blastn comparisons with three *S. agnetis* cattle isolates, 1379 (pink), 1387 (green), and 1385 (blue). Parameters were Query size = 10000, overlap 5000, expect=0.0001. Intensity of the color is indicative of the blastn score. The outer two rings show the annotated genes for isolate *S. agnetis* 908. The innermost ring indicates Mbp, the second most inner ring is the GC skew (magenta GC skew-; green GC skew+), and the third most inner ring plots GC content. The Numbered boxes indicate the locations of the 5 gene islands discussed in the







**Figure 2.5. CGView blastn comparison of *S. agnetis* 1379, CBMRN, 908 and 1416 genomes.** The *S. agnetis* 1379 2.4 Mbp chromosome was the reference for blastn comparisons with *S. agnetis* cattle isolate CBMRN (pink), and chicken isolates; 908 (green), and 1416 (blue). All other details are as in Figure 2.3 legend.

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## **CHAPTER 3**

### **Optimization of Factors and Conditions for Electroporation of the Emerging Pathogen**

*Staphylococcus agnetis.*

### **Chapter 3: Optimization of Factors and Conditions for Electroporation of the Emerging Pathogen *Staphylococcus agnetis*.**

#### **Abstract**

An electroporation procedure has been optimized for DNA transformation of *Staphylococcus agnetis*. We reported that chicken *S. agnetis* osteomyelitis isolates are closely related to cattle subclinical mastitis isolates, but we could not discern any particular genes as specific for the jump of *S. agnetis* from cattle to chickens. Molecular genetic investigations of this organism will facilitate identification of the determinants of virulence and host-specificity for this emerging pathogen. No transformation protocol has been described for *S. agnetis*. Therefore, we have optimized an electroporation method for DNA transformation so that we regularly obtain 10 to 20 transformants per ng using a Gram<sup>+</sup>/Gram<sup>-</sup> shuttle vector. The optimized protocol works on multiple different isolates of *S. agnetis* and provides a quick and reproducible method for molecular manipulation of this emerging pathogen.

**Running Title:** Electroporation of *S. agnetis*

#### **Importance**

*Staphylococcus agnetis* has been recently recognized as a significant pathogen in chickens and associated with mastitis in cattle. To better understand the biology of this emerging disease-causing bacterial species, we have developed a reliable method for introducing DNA for genetic manipulation.

**Keywords:** electroporation, *Staphylococcus agnetis*, transformation, pathogen.

## Introduction.

*Staphylococcus agnetis* is a coagulase-variable, Gram-positive bacterium originally identified with subclinical mastitis in cattle (1-3). More recently, this organism has been cultured from organs, blood, and bones in diseased chickens (4-6). Whole genome analyses have revealed that the chicken isolates derive from a single clade in the cattle isolates, but detailed genome comparisons failed to identify any particular virulence genes acquired or lost in the jump from cattle to chickens (6).

Conversely, genome comparisons of isolates of *Staphylococcus aureus* from humans and chickens had suggested that mobile genetic elements were likely associated with the change in host (7). Our research group has identified one particular chicken isolate, *S. agnetis* 908, that readily infects broiler chickens (4, 8, 9). Bacteria translocate across epithelial barriers and gain access to the blood (8, 10).

If bacteria can survive in the blood some will colonize of the growth plates of the proximal heads of the rapidly growing leg bones leading to necrosis known as bacterial chondronecrosis with osteomyelitis (BCO), one of the most prevalent forms of lameness in chickens and one of the most serious animal welfare issues in the industry (11). BCO-lameness has been associated with a number of different bacterial species (12-17).

However, there is little known about the determinants of host or tissue specificity. *S. agnetis* 908 appears to be highly adapted for these processes since administration of  $10^5$  CFU/ml in drinking water at 20 days of age leads to >50% BCO-lame broilers by 56 days of age (9, 18). This same isolate also triggers accumulation of double-stranded RNA in human and chicken bone osteoblasts leading to apoptosis (19). In order to further investigations of the virulence

determinants and host range determinants in *S. agnetis* we have optimized an electroporation system for transformation of this emerging pathogen.

## **Materials and Methods.**

### **Media.**

Media used in these investigations were: B2 (23, 24), Tryptic Soy Broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ), Luria–Bertani (per liter: 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl), and Brain Heart Infusion (Difco).

### **Bacterial cultures.**

*S. agnetis* isolates utilized included *S. agnetis* 908 isolated from a chicken BCO lesion at the University of Arkansas Poultry Research farm (4). *S. agnetis* 1379 and 1387 were isolated from dairy cow mammary gland/milk, and provided by Dr. John Middleton, University of Missouri. Genomes for these organisms have been deposited with NCBI and have been described (6). Stocks were maintained on tryptic soy agar slants (Becton, Dickinson and Company, Franklin Lakes, NJ) and archived at -80 °C in tryptic soy broth plus 40% glycerol.

### **Optimized preparation of electrocompetent cells and electroporation.**

Six ml of an overnight culture in B2 broth was diluted into 100 ml prewarmed B2 broth and grown at 37 °C with shaking to an OD<sub>660</sub> of 0.4-0.6. The flask was then swirled in an ice water slurry for 5 min. Cells were collected (all centrifugation was 2672 x g for 5 min, at 4 °C), and

washed by resuspension in 75 ml ice-cold sterile water, then pelleted. Cells were resuspended in 50 ml ice-cold 0.5 M sucrose and incubated on ice for 30 minutes. This was followed by centrifugation and resuspension in 15 ml ice-cold 0.5 M sucrose, and then in 3, 1.5 ml and finally 0.4 ml ice-cold EP (10% glycerol, 1.1 M sucrose). The final suspension was incubated on ice for 5 min, and aliquoted at 90  $\mu$ l. Cells were either used directly for electroporation, or stored at  $-80^{\circ}\text{C}$ . Frozen cells were thawed on ice for 10 min, incubated at room temperature for 25 min. For electroporation, the freshly prepared or thawed cell suspension, was transferred to a sterile 0.5 ml tube, and pelleted. The majority of the supernatant was aspirated and discarded, then the cells were resuspended by gently pipetting up and down. The tube was placed in a  $50^{\circ}\text{C}$  thermocycler block for 2 min, incubated at room temperature for 2 min, cells pelleted, and then resuspended in 400  $\mu$ l room temperature EP. The cells were pelleted, and then resuspended in 90  $\mu$ l room temperature EP, and incubated at room temperature for 30 min. The shuttle vector, plasmid pLI50 (20), was used for determining transformation frequency. The plasmid was isolated from *Escherichia coli* TB1 by standard alkaline SDS methods (29), and dissolved in sterile water. Plasmid was added (35 – 135 ng/ $\mu$ l) to the cells, mixed gently by pipetting, and the tube incubated at room temperature for 25 min before transferal to a 2 mm gap electroporation cuvette. Electroporation used a Gene Pulser (BioRad, Hercules, CA) programmed to 2 kV, 25  $\mu$ Fd, 200 Ohms. Time constants ranged from 3.8 - 4.4 ms. After electroporation, the cuvette was rinsed with 900  $\mu$ l  $37^{\circ}\text{C}$  B2 broth without antibiotics and the contents transferred to a 14 ml sterile culture tube, incubated 5 min at room temperature, and then at  $37^{\circ}\text{C}$  with shaking for 2 hours. Aliquots were plated on tryptic soy agar containing 12 $\mu$ g/ml chloramphenicol and incubated overnight at  $37^{\circ}\text{C}$  for plate counts and calculation of transformation efficiency.



## Results and Discussion.

For developing a transformation system, we used the Gram<sup>+</sup>/Gram<sup>-</sup> shuttle vector pLI50 (courtesy of M. Smeltzer, University of Arkansas for Medical Sciences) (20). A review of the literature identified a protocol for *S. carnosus* which included a heat treatment to temporarily inactivate restriction systems (21).

**Heat treatment to inactivate restriction system.** For *S. carnosus* they identified 2 minutes at 56 °C as the highest heat treatment without significant loss of viability. We therefore subjected *S. agnetis* 1379 to 2 minutes of heating using 2 degree steps from 46 to 60 °C. For 46, 48, 50, 52, 54, 56, and 58 °C (Table 3.1), transformation frequencies were recorded (Fig. 3.1). The maximum heat treatment was 50 °C for no loss of viability, while using 52 °C the viability was reduced by 50%.

Using the protocol of Löfblom *et al.* (21) with heat treatment of 50 °C, only 1 of 6 preparations produced any transformants and the transformation efficiency was 0.5 CFU/ng. Therefore, we investigated specific parameters to improve the reliability and efficiency of the protocol for *S. agnetis* (Table 3.2). The parameters we tested were primarily based on reports in the literature regarding other Gram-positive species.

**Growth media for preparing the log phase cultures.** We tested Tryptic Soy Broth, Luria–Bertani, B2, and Brain Heart Infusion growth media for preparing the log phase cultures (21). The transformation efficiencies for these media were compared (Fig. 3.2). Even though B2 was higher for transformation frequency, no significant difference was found.

**Centrifugation speeds.** We examined the effect of centrifugation speeds of 2672, 3181, 3732, 6381, and 9738 x g, on the transformation frequency (22). For these speeds, we recorded the transformation frequencies (Fig. 3.3). We found that centrifugation speeds higher than 2672 x g resulted in reductions of 90% or more in transformation efficiency.

**Temperature for the cell washes.** We investigated 4 °C versus room temperature (20 °C) for the cell washes (23, 24). Transformation frequencies were compared (Fig. 3.4). While RT was a little higher for transformation frequency, no significant difference was detected in transformation frequency.

**Sucrose for the cell wash.** We included a 30 minute soak in 0.5 M versus 1.1 M sucrose during the wash (25). We found the transformation frequencies of  $7.33 \pm 1.89$  CFU/ng for 0.5 M, versus  $10.5 \pm 2.54$  CFU/ng for 1.1 M sucrose (Fig. 3.5), which increased the transformation frequency by 30%.

**Incubation times of the cells with DNA at RT prior to electroporation.** Incubation of the mixture of cells and DNA at room temperature for 0, 10, 20, 25, and 30 min prior to electroporation (23). The transformation frequencies were compared (Fig. 3.6). This determined that the frequency increased by 16% with the longer incubation time.

**Cuvettes.** Comparison of 1.0 mm and 2.0 mm gap cuvettes (26). We found the frequencies of  $4.81 \pm 1.32$ , and  $13.5 \pm 2.90$  CFU/ng, respectively (Fig. 3.7). It indicated that 2.0 mm cuvettes resulted in an increase of 55% in transformation frequency.

**Voltage (kV) for the electroporation.** Comparison of 0.7, 1.75, and 2.0 kV for the electroporation (27). We compared the transformation frequencies (Fig. 3.8), which indicated that 2.0 kV resulted in an increase of 85 % in transformation frequency.

**Time constants.** Time constants varied from 2.7 to 4.4 milliseconds between preparations. For the recorded time constants, the transformation frequencies were reported (Fig. 3.9). We found few transformants when time constant is less than 3.8 milliseconds.

**Growth media for recovering the cells after electroporation.** We investigated antibiotic-free B2, Tryptic Soy Broth, and Luria–Bertani media for recovering the cells after electroporation (15). We recorded transformation frequencies (Fig. 3.10). We found that with B2 slightly higher for transformation frequency.

**Recovery-incubation times after electroporation.** We evaluated recovery-incubation times of 45, 60, 120, and 180 min. The transformation frequencies were compared (Fig. 3.11). We recovered few transformants at times shorter than 120 min.

**Comparison of fresh and frozen cells.** We compared fresh and frozen cells from the same preparation (28). The transformation frequencies were  $5.95 \pm 2.12$  CFUs/ng for fresh cells, and  $5.05 \pm 1.06$  CFUs/ng for frozen cells of the same cells preparation (Fig. 3.12). We determined that even after 3 months of storage at  $-80^{\circ}\text{C}$  the transformation frequency only decreased by 15 %.

**Reproducibility of the optimized protocol.** The final protocol, as presented in Materials and Methods, was repeated 35 times with *S. agnetis* 1379 and successfully produced competent cells 23 times with an average transformation frequency of  $16 \pm 3$  (s.e.m.) CFU/ng and a range of 0.3 to 55 CFU/ng. We also used this protocol to produce electrocompetent cells from *S. agnetis* 908 (4) and *S. agnetis* 1387 which, like 1379, was isolated from dairy cattle mastitis samples (6). Transformation frequencies using pLI50 were  $6 \pm 0.8$  CFU/ng for 908 and  $3.3 \pm 0.4$  CFU/ng for 1387 (Fig. 3.13), and the ranges were 0.25 to 11.75 CFU/ng for 908 and 0.2 to 6.6 CFU/ng for 1387.

We have deposited genomes for these and other *S. agnetis* isolates in NCBI (4, 6), and the transformation protocol we describe will be critical for dissecting critical questions concerning host-pathogen specificity, and tissue tropism. The differences between the initial protocol versus our optimized protocol are shown in Table 3.3. To the best of our knowledge, this is the first protocol optimized for transforming DNA into the cells of *S. agnetis* isolates.

**Table 3.1.** Determining the best temperature for heat induce the restriction system inactivation of the host cells of *S. agnetis*.

#	Temperature	CFU from 10 <sup>-6</sup> dilution	CFU from 10 <sup>-7</sup> dilution
1	46 °C	59	45
2	48 °C	65	69
3	50 °C	75	74
4	52 °C	29	27
5	54 °C	5	2
6	56 °C	2	0
7	58 °C	0	1
8	60 °C	0	0

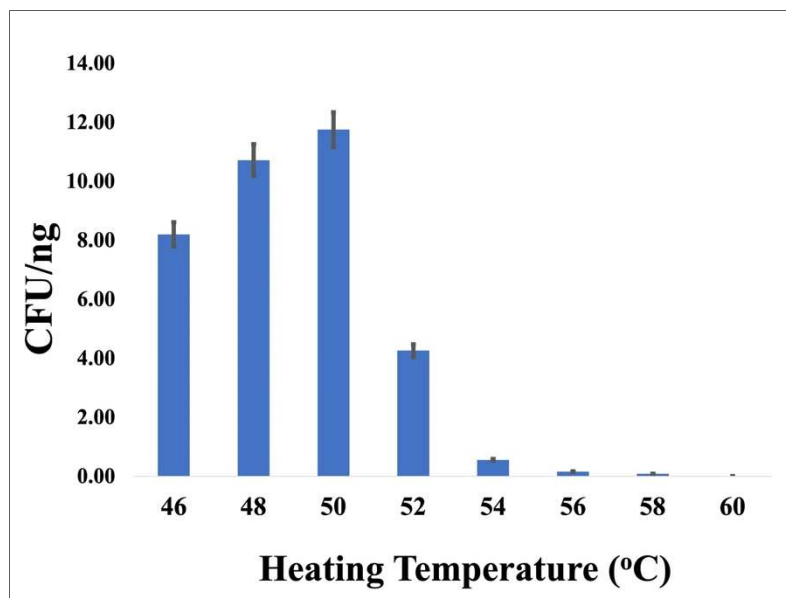
**Table 3.2.** Summary of the effects of evaluated different parameters on the transformation frequency for *S. agnetis*.

Step	Parameter	Effect*	Percent of change	Used	Literature
Electrocompetent cell preparation	Heat-treatment	+	50 %	46 - 60 °C	Löfblom et al. 2006
	Growth medium	0		TSB, LB, B2, BHI	Löfblom et al. 2006
	Centrifugation (x g)	–	90 %	>2672 x g	Schneewind and Missiakas 2014
	Washing Temperature (°C)	0			
	Freshly prepared cells	+	15 %		Keersmaecker et al. 2006
Treatments prior to electroporation	Sucrose (M)	+	30 %	0.5 - 1.1 M	Kraemer and Iandolo 1990
	Preincubation time (min)	+	16 %	0 - 30 min	Augustin and Götz 1990
Electroporation conditions	Cuvette (mm)	+	55 %	1 - 2 mm	Grosser and Richardson 2016
	Voltage (kV)	+	85 %	0.7 - 2.0 kV	Miller et al. 1988
	Time Constant	–	45 %	< 3.8 ms	Miller et al. 1988
Treatments post electroporation	Recovery medium	+	15%	B2	Löfblom et al. 2006
	Recovery-incubation time	–	80%	< 120 min	

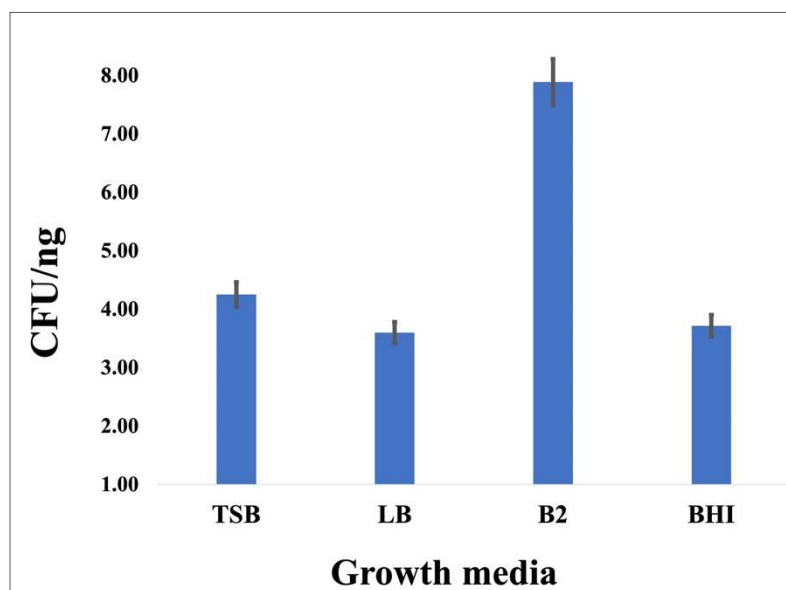
\*Influences on transformation frequency: + = positive effect; – = negative effect; 0 = no

**Table 3.3.** The early and optimized protocols used to transfer DNA to *Staphylococcus agnetis*.

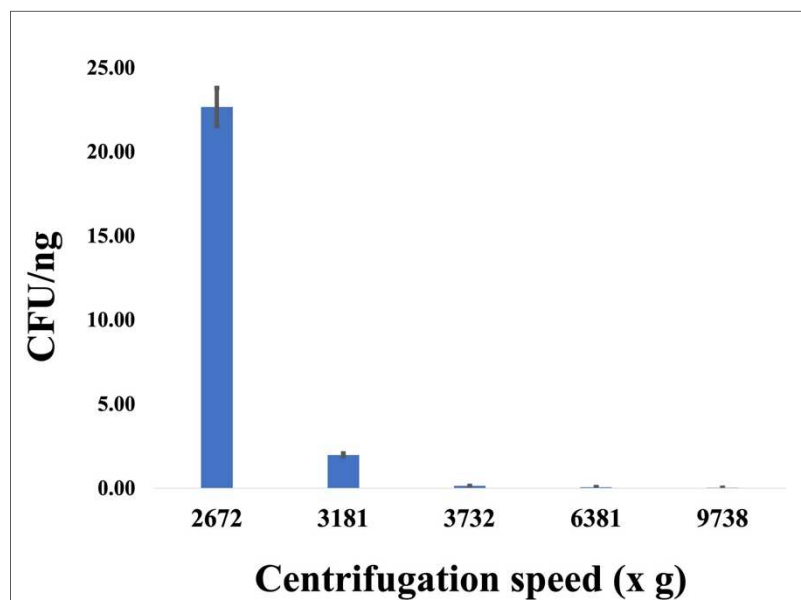
<b>Procedure step</b>	<b>Initial protocol</b>	<b>Optimized protocol</b>
Making electrocompetent cells	Growth from OD <sub>578</sub> 0.5-0.6	Growth from OD <sub>660</sub> 0.4-0.6
Cells thawing	5 min on ice, 10 min at RT	10 min on ice, 5 min at RT
Heat treatment	56 °C for 2 min then washed	50 °C for 2 min then washed
Centrifugation	3000 x g, 10 min and 4 °C	2672 x g, 5 min and 4 °C
Electroporation solution	0.5 M sucrose & 10% glycerol	1.1 M sucrose & 10% glycerol
Plasmid DNA amount	4000 ng	35 - 135 ng
Cuvette size	1.0 mm	2.0 mm
Electroporate Volts	21 kV	2.0 kV
Recovery	1 ml B2 broth, 37 °C, 48 hour	900 ul B2 medium, 37 °C, 2 hour
Transformation frequency	0.5 transformants/ng DNA	55 transformants/ng DNA



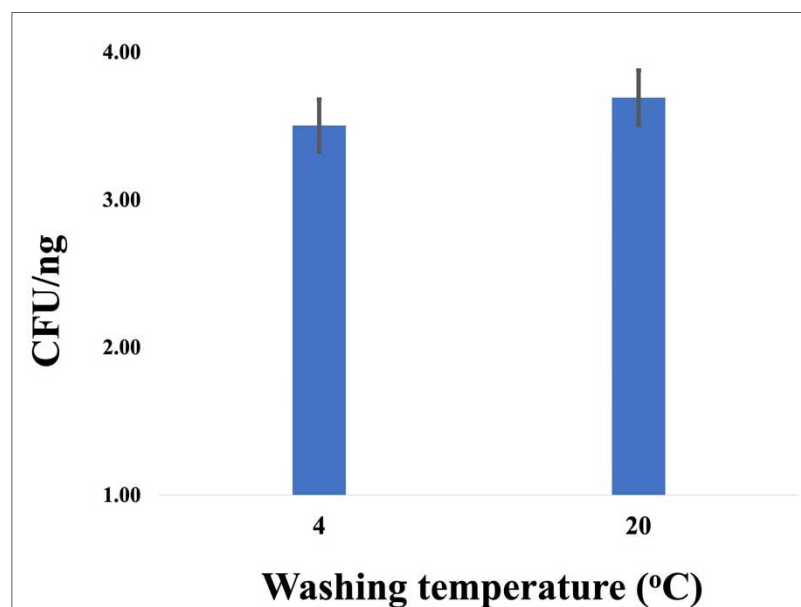
**Figure 3.1.** Effect of heat inactivation of restriction system on the transformation frequency.



**Figure 3.2.** Effect of four growth media (TSB, LB, B2, BHI) on the transformation frequency.

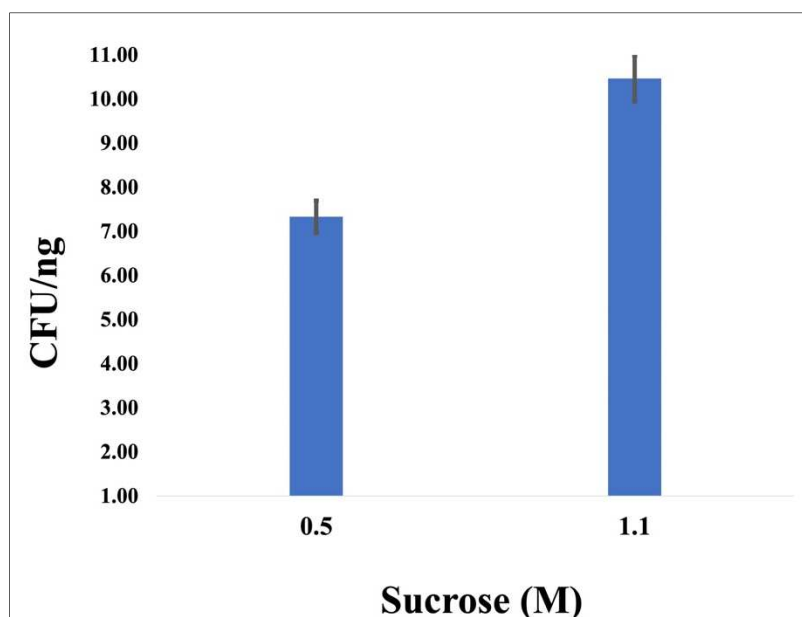


**Figure 3.3.** Effect of different centrifugation speed on the transformation frequency.

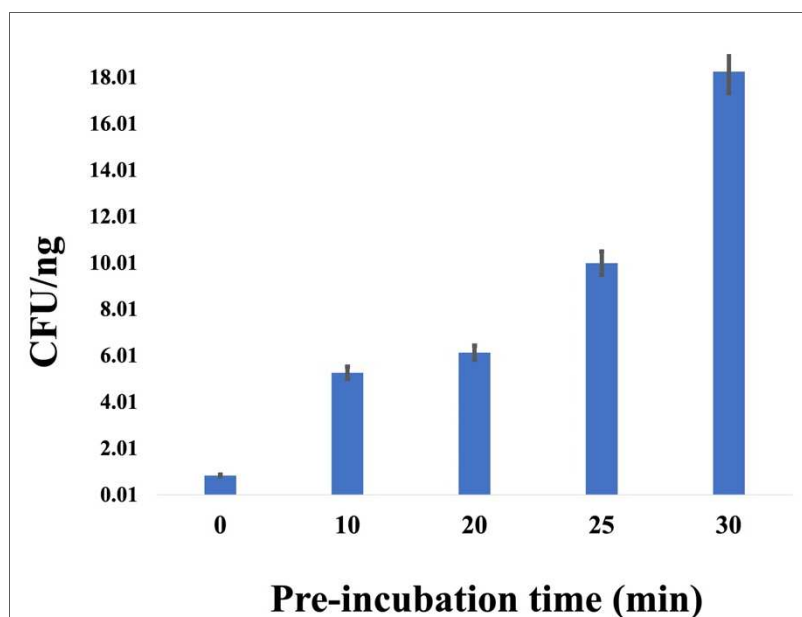


**Figure 3.4.** Effect of cell washes in 4 °C versus RT on the transformation frequency.

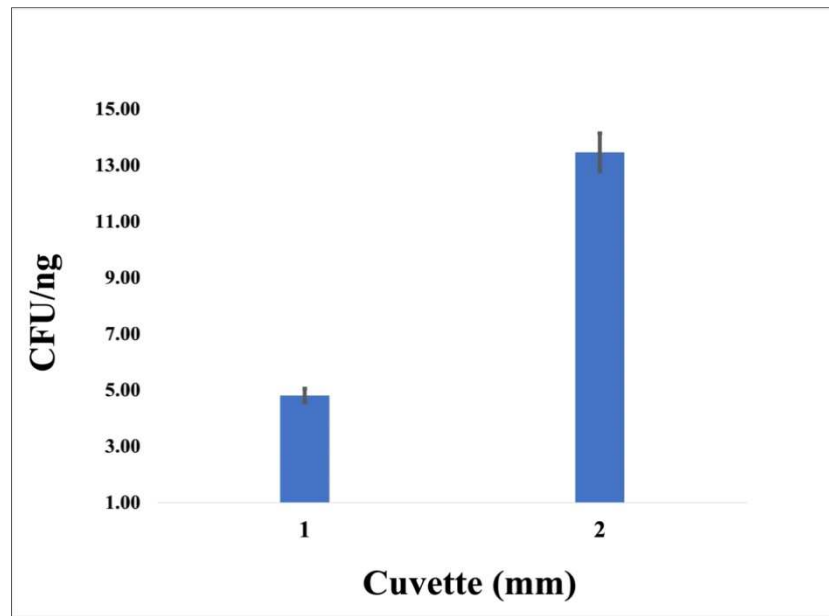




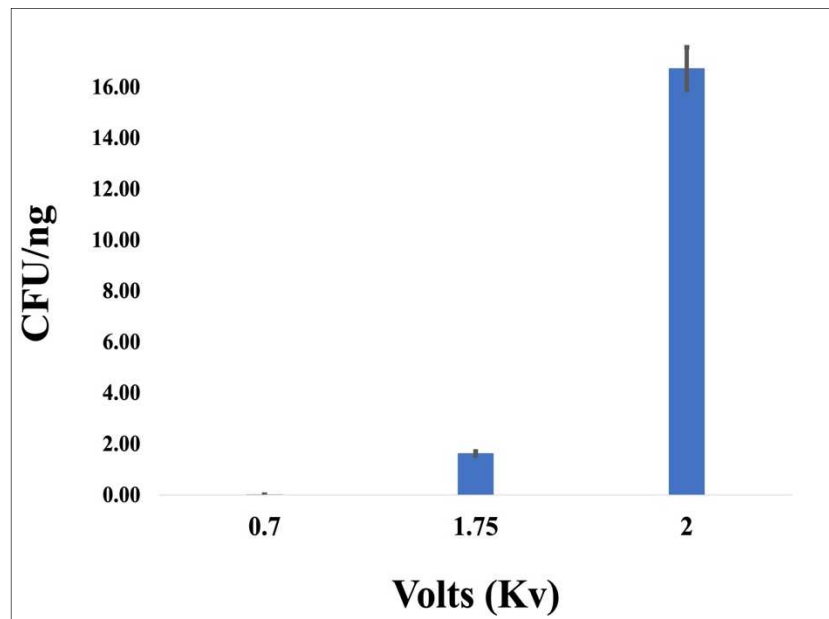
**Figure 3.5.** Effect of soak in 0.5 M vs 1.1 M Sucrose during the cell wash on the transformation frequency.



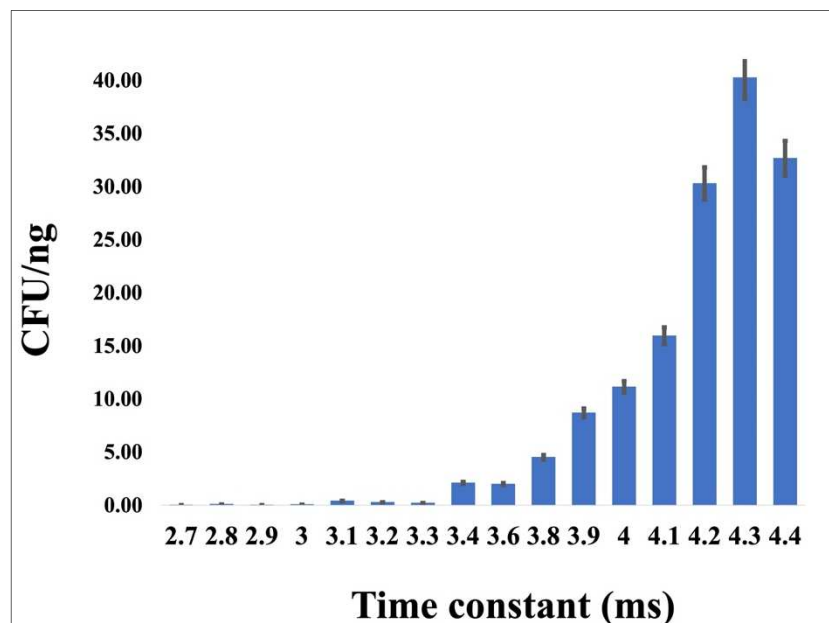
**Figure 3.6.** Pre-incubation time of the cells with DNA before electroporation and the transformants acquired per used plasmid DNA quantity (CFU/ng). Effect of 1.0 mm versus 2.0 mm gap cuvettes on the transformation frequency.



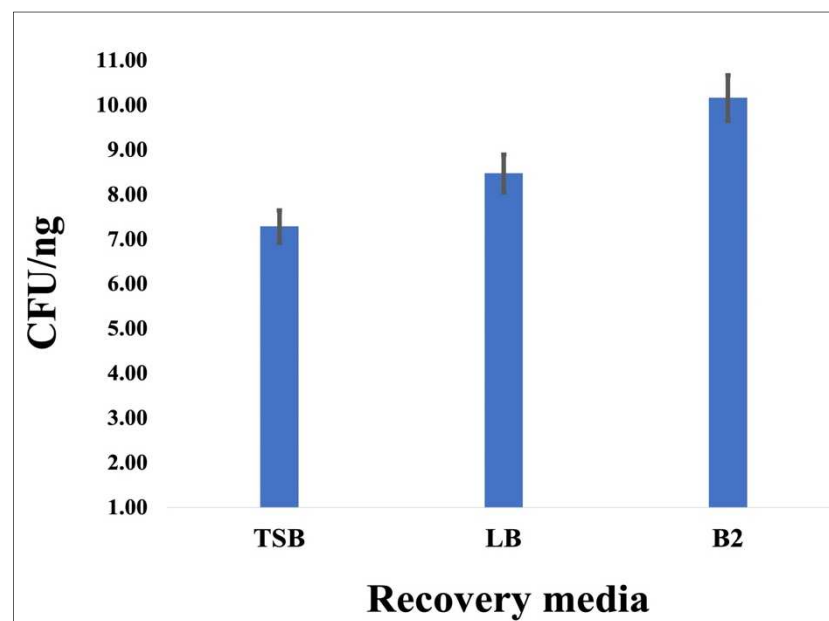
**Figure 3.7.** Effect of 1.0, versus 2.0 mm (Cuvette) on the transformation frequency.



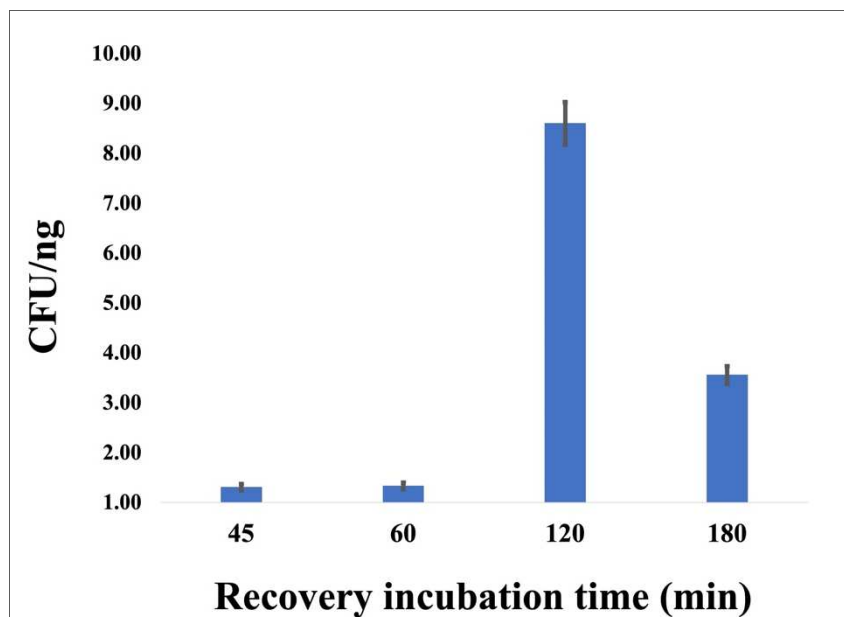
**Figure 3.8.** Effect of 0.7, 1.75, versus 2.0 volts (Kv) on the transformation frequency.



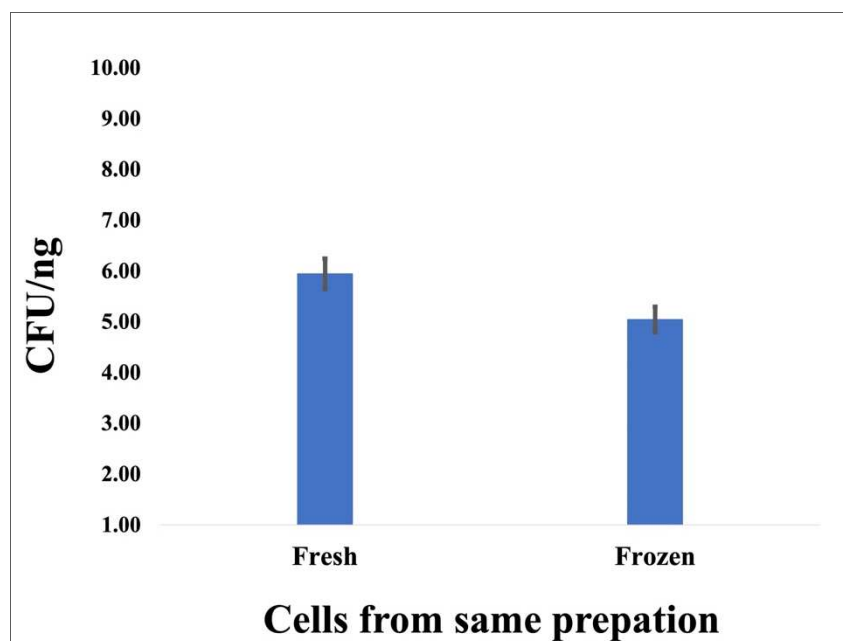
**Figure 3.9.** Time constant (ms) 2.7 to 4.4 and the transformation frequency.



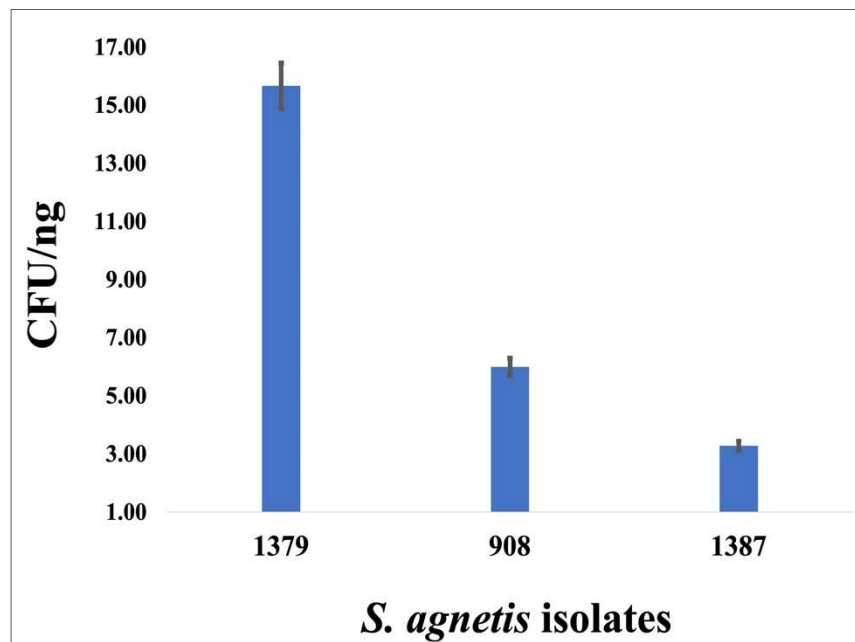
**Figure 3.10.** Effect of TSB, LB, and B2 recovery media on the transformation frequency.



**Figure 3.11.** Effect of 45, 60, 120, and 180 min recovery incubation time on the transformation frequency.



**Figure 3.12.** Effect of fresh and frozen cells from the same preparation on the transformation frequency.



**Figure 3.13.** Repetition of the final protocol on *S. agnetis* isolate 1379; 908; & 1387.

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## **CHAPTER 4**

**pfbA gene as a new genetic marker for *Staphylococcus* species identification.**

## Chapter 4: pfbA gene as a new genetic marker for *Staphylococcus* species identification.

### Abstract.

*Staphylococcus* species are the most common isolates from bacterial chondronecrosis with osteomyelitis (BCO) infected broilers worldwide. The aim of this study was to develop a reliable, rapid, highly sensitive quantitative PCR (qPCR) assay for identification of prevalence of different *Staphylococcus* species in BCO samples. The qPCR targets the gene plasmin and fibronectin-binding protein A (pfbA) gene which codes for a cell wall protein of 352 – 378 amino acids. A total of 762 *Staphylococcus* pfbA sequences were collected from NCBI. Phylogenetic trees based on pfbA gene revealed differentiation by species; consistent with whole genome comparisons. PCR primers were designed to specifically amplify the pfbA gene from select species. The qPCR protocol was confirmed *in silico* on 42 *Staphylococcus* species available in the NCBI databases. We empirically validated the protocol discriminates nine *Staphylococcus* species (*chromogenes*, *aureus*, *hyicus*, *cohnii*, *saprophyticus*, *agnetis*, *lentus*, *capitis*, and *epidermidis*) isolated from lame birds. The qPCR procedure described is suitable for quantifying these nine species from biological samples. Overall, the pfbA gene represents a suitable PCR target for species discrimination for Staphylococcal species.

## Introduction.

Bacterial chondronecrosis with osteomyelitis (BCO) in broilers is a bacterial disease caused by a range of bacteria including several species of *Staphylococcus* (Wideman and Prisby, 2013). The *Staphylococcus* genus comprises Gram-positive bacteria that inhabit the skin, gastrointestinal tract, mucosal membranes, and feces of animals, and can be found in environmental samples (Piette & Vandenberg 2009; Sampimon et al., 2009). Millions of dollars are lost annually in the broiler industry due to lameness caused by BCO; a serious animal welfare problem (Shwani et al., 2020; Al-Rubaye et al., 2017; Wideman and Prisby, 2013). In humans, *Staphylococcus* has been associated with common skin infections including respiratory infections, food poisoning, and abscesses. Staphylococci are spread by direct contact with an infected human or animal, by handling a contaminated item, or by breathing in contaminated droplets in air (Masalha et al., 2001).

*Staphylococcus* is the most common genus in the family Staphylococcaceae.

*Staphylococcus* includes 70 reported species. Around 60 *Staphylococcus* species have been found in broilers (Szafraniec et al., 2022; Lory 2014). Many *Staphylococcus* species are opportunistic pathogens of humans and animals, and induce infections of varying severity under certain situations, such as, disruption of the skin, diminished immunity (Kloos and Bannerman, 1994; Kocianova et al., 2005). Bacteria of the genus *Staphylococcus* cause BCO-induced lameness (Bradshaw et al., 2002). The coagulase test can be used to distinguish between various types of Staphylococci. Staphylococci that produce hemolytic coagulase are sometimes referred to as the pathogen *S. aureus*, which is considered a significant cause of BCO and mastitis, whereas coagulase-negative *Staphylococcus* are usually classed together as "minor" pathogens (National Mastitis Council 1999). The *Streptococcus* genus is mostly catalase-negative, and their

cell wall composition is different to that of Staphylococci. *Staphylococcus* species are salt tolerant and heat resistant (Wilkinson, 1997; Kloos and Lambe, 1991). There are two coagulase-variable staphylococcal species that have been related to BCO lameness in broilers and bovine mastitis: *Staphylococcus hyicus* and *Staphylococcus agnetis*, both of which are associated with BCO lameness and bovine mastitis. *S. hyicus* was the first staphylococcal species to be characterized as coagulase variable (Kloos and Schleifer, 1986). The pathogenic members of this genus are known for the formation of coagulase enzymes which clot blood. This distinguishes the coagulase positive strains, *S. aureus* (a human pathogen), and, *Staphylococcus intermedius* and *S. hyicus* (two animal pathogens). *Staphylococcus epidermidis* is coagulase-negative, while *S. agnetis*, and the species *chromogenes*, *cohnii*, *hominis*, *lentus*, and *xylosus*, are coagulase variable (Kloos and Musselwhite, 1975).

Identification of bacterial species at the species level is critical in a variety of fields, including microbiology, medicine, aquaculture, agriculture, and food safety (Marx 2016). Traditionally, bacterial identification is carried out by evaluating colony morphology on different culture media, antibiotic susceptibility, biochemical assays, phage susceptibility, killer toxin susceptibility, and/or serology. These often lack sufficient variation to discriminate closely related strains (Li et al., 2009). Traditional techniques can be time-consuming, labor-intensive, or expensive. Biochemical classification by the API 20 (API test; bioMérieux, Hazelwood, MO, USA), have a poor specificity for discriminating *S. hyicus* and *S. agnetis* in mastitis samples (Zadoks et al., 2009). In 2012, the species *S. agnetis* was identified as a separate species from cattle mastitis (Taponen et al., 2012). Previously, the majority of coagulase-positive non-aureus *Staphylococcus* spp. were mostly identified as *S. hyicus* (Roberson et al., 1996). Biochemical assays employed to separate staphylococcal species resulted in incorrect classifications of the

bacteria. *S. agnetis*, *S. chromogenes* and *S. hyicus* were not identified as different staphylococcal species, until 1986 (Hájek et al., 1986), since they are highly similar, it was hard to tell them apart (Adkins et al., 2017). Development of rapid and accurate methods for staphylococcal identification are therefore warranted (Rebroová et al., 2017).

Molecular based approaches for distinguishing Staphylococcal species are preferable to biochemical methods (Zadoks & Watts 2009). Analysis of the 16S rDNA gene sequence is the most popular approach currently available, in the identification and classification of bacteria, (Becker et al., 2004). Based on 16S rDNA gene sequences, phylogenetic studies of members of the Staphylococcaceae family revealed the existence of paraphyletic and polyphyletic genera. Therefore, based on 16S rDNA, five *Staphylococcus* species, *S. fleurettii*, *S. lentus*, *S. sciuri*, *S. stepanovicii*, and *S. vitulinus*, were taxonomically re-assigned to *Mammaliicoccus* gen. nov. (Madhaiyan et al., 2020). However, when applied to other staphylococcal species, the 16S rDNA gene sequence is too similar for species discrimination (Gribaldo et al., 1997; Becker et al., 2004). For example, *S. agnetis* isolates had 99.1% and 97.7% similarity to those of *S. chromogenes* ATCC 43764 and *S. hyicus* ATCC 11249T, respectively (Taponen et al., 2012). Others have used alternative gene sequences for species discrimination, including *rpoB* (Drancourt & Raoult 2002), *tuf* (Heikenset al., 2005), *aroD* (Adkins et al., 2017), and *cpn60* (Kwok et al., 1996). Some have suggested that species cutoff values of 98.7% sequence (Jousson et al., 2007). Many genes have been suggested for typing and identification of different *Staphylococcus* species, such as 16S rRNA, *rpoB*, *hsp60*, *aroD*, *sodA*, *tuf*, *ackA*, *fdhD*, *fdhF*, *groEL*, *purA*, *tpiA*, and whole-genome sequencing (Adkins et al., 2017; Mellmann et al. 2006, Hwang et al., 2011, Naushad et al., 2016, Shwani et al., 2020). Most of the sequences of the *rpoB* genes in *S. agnetis* and *S. hyicus* strains from cows are almost the same. The two closely

related species of *S. agnetis* and *S. hyicus* could be distinguished from each other using *rpoB* since these isolates share only 93.5% similarity (Mellmann et al. 2006). *Staphylococcus* species, such as *S. agnetis* and *S. hyicus*, can be distinguished using the *tuf* and *aroD* genes (Hwang et al., 2011). In addition, multiple core genes, such as 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* genes, were concatenated all together to type *S. agnetis*, *S. arlettae*, *S. auricularis*, *S. capitis*, *S. caprae*, *S. chromogenes*, *S. cohnii*, *S. devriesei*, *S. equorum*, *S. gallinarum*, *S. hominis*, *S. hyicus*, *S. kloosii*, *S. nepalensis*, *S. pasteurii*, *S. saprophyticus*, *S. sciuri*, *S. simulans*, *S. succinus*, *S. vitulinus*, *S. warneri*, and *S. xylosus* (Naushad et al., 2016). In our previous study, we built phylogenetic trees based on seven housekeeping genes (*ackA*, *fdhD*, *fdhF*, *groEL*, *purA*, *tpiA*, and *tuf*) concatenated to one another (Shwani et al., 2020). Moreover, whole genome sequencing (WGS) was also used for typing of the clinically important *S. aureus* (Humphreys & Coleman, 2019; Tsang et al., 2017). But, many gene sequencing methods may be required before a correct identification of a staphylococcal isolate can be achieved. Therefore, these approaches are reliable typing approaches, but they make the process very tedious, expensive, and time consuming.

However, these genes above can sometimes misidentify *Staphylococcus* species; for example, *S. equorum* cannot be distinguished using partial *rpoB* (Mellmann et al. 2006). Also, using 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* genes, the genomes PYYE01.1 and QXSE01.1 (Naushad et al., 2016) were misidentified as *S. fleurettii* SNUC\_248 and *S. fleurettii* SNUC\_248, respectively. We could correct them and reassign them to their correct taxonomic positions, which are *S. vitulinus* SNUC\_248 and *S. vitulinus* SNUC\_248, respectively.

A reliable, fast, and cheaper method to discriminate between different *Staphylococcus* can improve the identification of different *Staphylococcus* isolated from lame birds. We have

published whole genome sequences of *S. agnetis*, *S. hyicus*, *S. aureus*, and *S. chromogenes* (Shwani et al., 2020; Ekesi 2020; Alrubaey et al. 2015). When the whole genome sequences of *S. agnetis*, *S. hyicus*, *S. aureus*, and *S. chromogenes* were examined, we revealed distinct gene sequences, such as plasmin and fibronectin-binding protein A (pfbA) gene, that were believed to be suitable discriminating sequences for identifying these species and other species of *Staphylococcus* as well when employing cPCR and qPCR platforms.

The pfbA gene is 1100 bp and encodes a cell wall protein. This gene encodes for a cell wall protein consisting of 352–378 amino acids. The pfbA protein was not described in detail in *Staphylococcus*. We did not find any literature to cover the conserved and variable regions of this polypeptide, nor what the polypeptide does in virulence. However, pfbA gene was described in *Streptococcus*. Sometimes, it is also known as pectate lyase (pelL) in the NCBI. This protein was identified on the surface of *Streptococcus pneumoniae* as a conserved surface protein that supports the bacterium in colonizing its host by recognizing the extracellular matrix molecule fibronectin and other factors in the blood such as plasminogen and human serum albumin (Beulin et al., 2017).

The goal of this study was to establish a simple, cheap, and quick quantitative PCR approach for the discrimination and typing of a collection of *Staphylococcus* spp. We included 42 species of the *Staphylococcus* genus with 762 isolates in silico investigation. For this purpose, species specific pfbA primers were designed and used for 9 species of *Staphylococcus* in both conventional PCR (cPCR) and quantitative PCR (qPCR) experiments. The approach was empirically tested on *Staphylococcus chromogenes*, *S. aureus*, *S. hyicus*, *S. cohnii*, *S. saprophyticus*, *S. agnetis*, *S. lentus*, *S. capitis*, and *S. epidermidis* isolated from BCO lame birds

at the University of Arkansas poultry research facilities and cattle milk, mammary gland secretions, or udder skin at the University of Missouri.

This could result in the establishment of a massive reference database library for the *pfbA* typing technology, which could then be used to determine the identity of any unknown bacterial sample. As far as we are aware, the *pfbA* gene was never used to diagnose any species belonging to *Staphylococcus*.

## **Materials and Methods.**

**Reference genomes.** Genome sequences for 762 isolates from 42 *Staphylococcus* species were downloaded from the National Center for Biotechnology Information (NCBI) database.

Accession numbers, host sources, and citations are provided in Table 4.1.

**Bacterial strains.** Eight *Staphylococcus* species were from a collection of previously isolated from lame birds at the University of Arkansas poultry research facilities. The identification of the nine *Staphylococcus* species used in this study was confirmed based on CHROMagar media (Fig. 4.1), and 16S rDNA gene sequence, or partial DNA sequence of either elongation factor Tu (*tuf*) or 3-dehydroquinate dehydratase (*aroD*) analysis (Alrubaye et al., 2015, Adkins et al., 2017; Shwani et al., 2020). The bacterial strains were including *S. chromogenes* isolate 1401 (Shwani et al., 2020), *S. agnetis* isolate 908 (Alrubaye et al., 2015), *S. aureus* isolate 1516 (Ekesi 2020), *S. cohnii* isolate 1561, *S. saprophyticus* isolate 876, *S. lentus* isolate 1559, *S. capitis* isolate 1557, and *S. epidermidis* isolate 886. Also, *S. hyicus* isolate 1381 was from a collection at the



University of Missouri isolated from cattle milk, mammary gland secretions, or udder skin (Shwani et al., 2020). Thus, we had 9 *Staphylococcus* species to work with in this study.

**Bacterial cultures and DNA extraction.** The bacterial cultures and DNA extraction were performed according to Shwani et al. (2020).

**Designing PCR primers.** We chose the pfbA gene as a novel gene for *Staphylococcus* species typing. Plasmin and fibronectin-binding protein A (pfbA) forward and reverse PCR primers (pfbA-FxR) were designed for the 9 *Staphylococcus* species used in this study for validation of our in silico results. The species are *Staphylococcus chromogenes* (1401), *S. aureus* (1516), *S. hyicus* (1381), *S. cohnii* (1561), *S. saprophyticus* (876), *S. agnetis* (908), *S. lentus* (1559), *S. capitis* (1557), and *S. epidermidis* (886), for which the primers were named chr\_pfbA-FxR, aur\_pfbA-FxR, hyi\_pfbA-FxR, coh\_pfbA-FxR, \_sap pfbA-FxR, agn\_pfbA-FxR, lent\_pfbA-FxR, cap\_pfbA-FxR, and epi\_pfbA-FxR, respectively (Table 4.4). We have prepared a number of mixtures of these pfbA primers. The 9\_pfbA-FxR (5 uM) contains an equal amount of all nine *Staphylococcus* species pfbA-FxR (10 uM) primers. Each mixture includes equal portions of only 3 *Staphylococcus* species pfbA-FxR primers. A\_Mix includes chr, coh, and lent pfbA primers. B\_Mix includes aur, sap, and cap pfbA primers. C\_Mix includes hyi, agn, and epi pfbA primers. D\_Mix includes coh, agn, and lent pfbA primers.

### **Conventional PCR assays for identification of 9 *Staphylococcus* species.**

We have designed sets of pfbA primers for 42 *Staphylococcus* species (Table 4.3), including the 9 pfbA primers above. The PCR reactions were performed in a total reaction volume of 20  $\mu$ l containing 2  $\mu$ l of Taq Buffer (10X), 0.2  $\mu$ l of dNTPs (20 mM), 0.5  $\mu$ l of *Staphylococcus* pfbA primers (10  $\mu$ M), 0.05  $\mu$ l of Taq Polymerase (80U/ $\mu$ l), and 1.0  $\mu$ l DNA extract. The reactions were achieved on a BioRad PCR machine (Bio-Rad Laboratories, Inc. USA) according to the following thermocycler conditions: 30 s at 90 °C, followed by 30 cycles of 15 s at 90 °C and 1 min at 60 °C, and extension at 72 °C for 60 s. Negative (No DNA) controls were also investigated for each PCR run.

**Quantitative PCR High Resolution Melting (HRM) Analysis.** In quantitative PCR, we have also analyzed the HRM for each *Staphylococcus* species. Using 96-well plates and the CFX thermal cycler, twenty microliter PCR reactions were carried out in 2 hours (BioRad, Hercules, CA). The qPCR master mix included 2  $\mu$ l of 10X buffer with 20 mM of MgCl<sub>2</sub>, 0.2  $\mu$ l of 20 mM dNTP, 0.5  $\mu$ l of 10  $\mu$ M forward and reverse pfbA primers, 1  $\mu$ l of 10x fluorescent dye EvaGreen (Biotium Inc., Fremont, CA), 0.05  $\mu$ l of 80 U Taq polymerase, and 1  $\mu$ l of bacterial DNA. The reactions were achieved on a CFX96 Touch quantitative PCR Detection System (Bio-Rad Laboratories, Inc. USA) according to the following thermocycler conditions: initial denaturation was 30 s at 90 °C, 5 cycles of denaturation at 90 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 60 s, followed by 30 cycles using the same parameters with a plate read after each cycle. In the following step, a High Resolution Melt (HRM) was performed, which consisted of heating samples to 72° C for 180 s, then cooling them to 60° C for 180 s. Then, fluorescence was measured during a temperature increase from 75 to 90 °C with 0.1 °C per five-

second increments, and the plate was read. The melt profiles of the extracted DNAs from three separate trials of the same tested species were compared. The Bio-Rad CFX management software was used to examine the melt curves that were acquired. Negative controls were investigated for each PCR run.

**Investigating the limit of detection.** Different DNA mixtures (0.25 ng/ul) of all the 9 *Staphylococcus* species were used. Also, DNA mixtures of only three species, in different ratios (80:10:10) were used, such as A\_DNA\_Mix (80 ul *S. cohnii*, 10 ul *S. agnetis*, and 10 ul *S. lentus* DNA (0.25 ng/ul.)), B\_DNA\_Mix (10 ul *S. cohnii*, 80 ul *S. agnetis*, and 10 ul *S. lentus* DNA), and C\_DNA\_Mix (10 ul *S. cohnii*, 10 ul *S. agnetis*, and 80 ul *S. lentus* DNA). The DNA mixes were then subjected to a couple of successive 10-fold dilutions. Then, only 1 ul of the diluted mixtures was used in quantitative PCR (or conventional PCR) reactions of 20 ul volume. The initial 1 microliter DNA template of the 3 bacteria consisted of 2: 0.25 : 0.25; and 0.25: 2: 0.25; and 0.25: 0.25: 2 picograms, respectively.

**Gel electrophoresis.** Following conventional and/or quantitative PCR, a 1.5% agarose gel electrophoresis was performed for 2 hours using 0.5% TBE buffer and voltages greater than 100 V. Then, the gel images were taken using a phosphorimager of a GE Typhoon<sup>TM</sup> FLA 9500 scanner (GE Health Care), in which we used a pixel size of 100 uM and 400 V (voltage) to be applied to the photo-multiplier tube (PMT). The size of the PCR product was determined using a 100-bp (or 1-kb) ladder. The gel images were analyzed using ImageJ software.

### **Phylogenetic analyses, pfbA genes and whole genome phylogenetic comparison.**

The genomes obtained from NCBI were further processed for building phylogenetic trees using the following two approaches. First, pfbA gene sequences and building multilocus sequence typing (MLST) phylogenetic trees. Second, acquiring the complete or whole genome sequences, building distance matrices, and then converting the matrices to phylogenetic trees. BLASTN searches were run for all of the genomes to collect all of the 762 pfbA gene sequences. For MLST investigation, MegAlign (DNASar) was used to align gene coding sequences of pfbA genes. The phylogenetic trees were generated using the Clustal Omega algorithm implemented in MegAlignPro (DNASar). Based on the alignments, consensus neighbor-joining trees with 2,500 bootstrap replications were generated. Also, we used the genome-to-genome distance calculator (GGDC; <http://ggdc.dsmz.de/ggdc.php>) to generate a phylogenetic tree based on genetic distances computed from whole-genome BLASTN comparisons (Meier-Kolthoff et al., 2013).

### **Anotation of some pfbA genes.**

Annotation of the pfbA genes was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) website (<https://www.genome.jp/kegg/>) (Morishima et al., 2019).

## **Results.**

### ***Staphylococcus* species genomes.**

The sources of all of the *Staphylococcus* isolates used in this study are found in Table 4.1. A total of 762 plasmin and fibronectin-binding protein A (pfbA) nucleotide sequences of 42 *Staphylococcus* species were collected for this study from the NCBI database. We have

previously disseminated the whole genome sequence of the chicken isolate *S. agnetis* 908 (Alrubaye et al., 2015), the draft genomes of *S. chromogenesis* isolate 1401, and *S. hyicus* isolate 1381 (Shwani et al., 2020), and deposited the *S. aureus* isolate 1516 (Ekesi 2020) in NCBI (unpublished). Also, we have isolated *S. cohnii* isolate 1561, *S. saprophyticus* isolate 876, *S. lentus* isolate 1559, *S. capitis* isolate 1557, and *S. epidermidis* isolate 886 from lame birds at the University of Arkansas poultry research facilities. These nine *Staphylococcus* species have been used in the current study to empirically validate the in silico results of this study. For the *Staphylococcus* pfbA gene sequences in NCBI that were not annotated, we used the KEGG website for annotation (Table S4.2).

### **Phylogenetic studies.**

In order to start to trace the phylogenomic association between nine *Staphylococcus* species isolated from broilers with BCO lameness; *S. chromogenesis* isolate 1401 (Shwani et al., 2020), *S. agnetis* isolate 908 (Alrubaye et al., 2015), *S. aureus* isolate 1516 (Ekesi 2020), *S. cohnii* isolate 1561, *S. saprophyticus* isolate 876, *S. lentus* isolate 1559, *S. capitis* isolate 1557, and *S. epidermidis* isolate 886, we compared a number of housekeeping and virulence ortholog genes (Data not shown). We found plasmin and fibronectin-binding protein A (pfbA) as the best candidate, which is an ortholog gene in all of the isolates and possesses a wide range of hypervariable regions between species, subspecies, and isolates of *Staphylococcus*. First, we constructed MLST phylogenetic trees using the pfbA genes of these nine species.

Since the pfbA gene is an ortholog that could be identified in any *Staphylococcus* species, we have included many species, subspecies, and isolates that belong to the *Staphylococcus* genus. The total species was 42, and 762 isolates (Table 4.1) have been subjected to pfbA MLST

analyses and constructed trees. The phylogenetic tree based on pfbA MLST analyses (Fig. 4.2) shows clear separation between different species, subspecies, and isolates of 42 different *Staphylococcus* species. Later, the whole genome sequence of these 762 isolates was subjected to distance matrices generation, which was then transferred to a distance phylogenetic tree to confirm the results of the pfbA phylogenomic tree. From the MLST analysis, we see that the *Staphylococcus* species are clearly separated from each other with  $\geq 89\%$  identity, with  $\leq 10\%$  separation between the subspecies belonging to the same species, and  $\geq 20\%$  separation between different species (Table S4.1). The phylogenetic tree built based on genetic distances derived from whole-genome BLASTN comparisons was generated using the GGDC distance calculator (Meier-Kolthoff et al., 2013) (Fig. 4.3) shows a topology and clades that are very similar to the tree built using MLST analysis for the pfbA sequences only. We also investigated pfbA protein sequences from all 42 *Staphylococcus* species and obtained the same phylogenomic architecture (Fig. 4.4) as those from the other phylogenomic trees (Fig. 4.1 & 4.2).

### **PCRs and gel images.**

Each of the nine *Staphylococcus* species that have been empirically checked has a separate pfbA band on gel electrophoresis based on the size of the amplicon.

### **The detection limit of the pfbA primers.**

For detection limits, our pfbA primers were used to amplify the DNA mixtures of A\_DNA\_Mix, B\_DNA\_Mix, and C\_DNA\_Mix. The PCR products run on 1.5% gel electrophoresis show the distinct separation of the 3 *Staphylococcus* species (Fig. 4.11 & 4.12).

### **The development of qPCR HRM curve analysis.**

We designed two sets of *pfbA* primers for each of the 42 *Staphylococcus* species (Table 4.3). These primers were made to obtain different-sized amplicons that can be seen on gel electrophoresis. We confirmed the applicability of this technique for discriminating between these eight different *Staphylococcus* species. We have tested these *pfbA* primers using different *Staphylococcus* species collected from BCO lesions at the University of Arkansas poultry research farm, plus a *Staphylococcus* species from cattle, as reference isolates. The reference bacterial species included *S. chromogenes*, *S. aureus*, *S. hyicus*, *S. cohnii*, *S. saprophyticus*, *S. agnetis*, *S. lentus*, *S. capitis*, and *S. epidermidis*. These species were previously determined to species level based on CHROMagar media (Fig. 4.1) and sequencing of the 16S rDNA gene (Shwani et al., 2020; Adkins et al., 2017, & Al-Rubaye, 2013). For these 9 species, we used *chr\_pfbA-FxR*, *aur\_pfbA-FxR*, *hyi\_pfbA-FxR*, *coh\_pfbA-FxR*, *sap\_pfbA-FxR*, *agn\_pfbA-FxR*, *len\_pfbA-FxR*, *cap\_pfbA-FxR*, and *epi\_pfbA-FxR* primers. The amplicons were 1027, 994, 905, 888, 836, 748, 673, 611, and 474 bp in size, respectively (Table 4.4).

Amplifications of *pfbA* genes were carried out using individual species specific *pfbA* primers, three distinct sets of *pfbA* primers, which were A\_Mix, B\_Mix, and C\_Mix of *pfbA-FxR* primers (10uM), and 9\_*pfbA-FxR* (5 uM) which includes all of the 9 *pfbA* primers. The PCR products were run on gel electrophoresis (Fig. 4.9–4.11), and from melting profiles, with EvaGreen showing a unique diagnostic profile for every species (Fig. 4.14).

## Discussion.

As far as we are aware, this is the first study to use the plasmin and fibronectin-binding protein A (pfbA) genes for *Staphylococcus* species, subspecies, and strain identification. We employed the pfbA gene for the first time for amplification and subsequent analysis to develop a rapid qPCR HRM assay. We examined nine pfbA template sizes to obtain distinct amplicons.

The pfbA gene in *S. pneumoniae* encodes for a cell wall protein that has an LPXTG anchoring motif and is capable of binding to human serum proteins. They looked into the involvement of pfbA in the etiology of pneumococcal disease. According to the findings of their research, the protein pfbA plays a key role in the development of pneumococcal infections. According to the results of the phylogenetic study, the pfbA gene in *S. pneumoniae* is highly conserved (Yamaguchii et al., 2008, 2015, 2019).

The goal of this investigation was to create a rapid and accurate molecular diagnostic technique for the detection and identification of *Staphylococcus* species retrieved from BCO lesions in lame birds. Species identification will take place in less than three hours after the broth or colonies have been collected. Our comparative analysis of the high resolution melting (HRM) profiles revealed that the different Staphylococcal species could be easily identified using species-specific pfbA primers as the most appropriate assay (Fig. 4.11, 4.12, 4.14 & 4.16).

In our in silico investigation, we used 762 pfbA gene sequences collected from the NCBI database belonging to 42 different *Staphylococcus* species to cover most of the *Staphylococcus* family. A number of these pfbA sequences collected from NCBI were not annotated. A confirmation annotation of these pfbA genes was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) website (<https://www.genome.jp/kegg/>) (Morishima et al., 2019) (Table S4.2).



Using species-specific primers that amplify the target pfbA gene, resulting in different amplicon sizes, is the best strategy in the HRM analysis because it provides different GC content, which leads to unique HRM profiles. In this research, we amplified the pfbA genes of different *Staphylococcus* species, and we successfully separated the species based on unique HRM curves and then ran the PCR products on gel electrophoresis. The different sized pfbA amplicons can be easily identified using agarose gel electrophoresis, as the most successful method of separating DNA fragments of varied sizes (Lee et al., 2012). This approach was a successful typing method to distinguish between different *Staphylococcus* species.

In our in silico study, we looked at a lot of different housekeeping and virulence genes and found that pfbA is a very important gene in *Staphylococcus* species. Yamaguchii et al., (2008) identified and named the pfbA (plasmin- and fibronectin-binding protein A) gene in *Streptococcus pneumoniae*, which encodes a cell wall protein with an LPXTG anchoring motif in *S. pneumoniae* that is bound to human serum proteins. They investigated the role of pfbA in pneumococcal pathogenesis and discovered that pfbA plays a significant role in the development of pneumococcal infections, and phylogenetic analysis indicated that the pfbA gene is highly conserved in *S. pneumoniae* (Yamaguchii et al., 2008, 2015, & 2019). Our results regarding the highly conserved pfbA as an ortholog in *Staphylococcus* species came in agreement with the findings Yamaguchii et al. (2008 & 2019) found in *S. pneumoniae*.

The pfbA analysis determined that the subspecies of the same *Staphylococcus* species have pfbA sequences with  $\geq 89$ –98% identity,  $< 10\%$  separation between sub species, and  $> 20\%$  separation across species (Table S4.1). This indicates that we can use just the pfbA gene on its own to type *Staphylococcus* species and place them in the right taxonomic group instead of

having to combine a number of housekeeping and virulence genes, or the whole genome sequences.

Performing a High Resolution Melt (HRM) and gel electrophoresis, we empirically investigated the nine different sizes of the *pfbA* templates that belong to nine different *Staphylococcus* species. In total, we obtained 9 different sized amplicons using *pfbA* primers for the 9 *Staphylococcus* species. This led to the facilitation of the identification of the *Staphylococcus* species based on their unique HRM curves and/or their different bands on 1.5% gel electrophoresis. The typing is performed in a short period of time (2–3 hours) and without the need to send the PCR products for sequencing. We can run the PCR products on 1.5% gel electrophoresis. The PCR *pfbA* primers designed based on different PCR amplicon sizes efficiently separate different *Staphylococcus* species on gel electrophoresis which saves times and efforts comparing to purification of the DNA or PCR products and sending for sequencing (Table 4.3). At the concentrations utilized in this comparative analysis, all nine *Staphylococcus* species produced PCR amplicons and HRM curves that were distinct from one another. All of the 9 DNAs from reference bacteria were amplified, and we obtained separate melt curve profiles for each *Staphylococcus* species tested in this study. There was a single peak in the melting profile of each of the nine species, each indicating a particular bacterial species. This was true for all nine species. On the gel electrophoresis, the 9 species have been identified based on different amplicon sizes.

Comparing the melt profiles of extracted DNA from three independent trials of the same tested species revealed no significant differences and confirmed the qPCR assay's repeatability. Negative controls were checked for each PCR run to rule out contamination in the reaction mixture. After a few 10-fold dilutions of the A\_DNA\_Mix, B\_DNA\_Mix, and C\_DNA\_Mix,

and using only 1 ul of the dilutions, the *Staphylococcus* species specific pfbA primers were able to successfully amplify the right species pfbA sequence (Fig. 4.11 & 4.12). Therefore, the detection limit of the primers is as low as 0.25 pg or even less. However, we did not check less than 0.25 pg in this study. Hence, the use of *Staphylococcus* pfbA primers to amplify the pfbA gene is the most accurate test for discriminating and typing *Staphylococcus* species.

The genes, such as 16S rRNA, rpoB, hsp60, aroD, and sodA (Mellmann et al., 2006, Hwang et al., 2011, Naushad et al., 2016, Adkins et al., 2017), have been used for identifying and discriminating different *Staphylococcus* species. However, the PCR assays they used were not confident in separating many closely related *Staphylococcus* species, such as *S. chromogenes*, a close species to *S. agnetis* and *S. hyicus*. Using pfbA gene we could easily separate the species easily. In our study, we found that the pfbA gene within all of the *Staphylococcus* species is very conserved and discriminating in telling apart different species, subspecies, and isolates of different *Staphylococcus* species.

The pfbA gene can be used to resolve the taxonomic issues and reassign *Staphylococcus* species to the right taxonomic positions. Also, pfbA is the best genetic marker to differentiate between different subspecies and isolates of the same *Staphylococcus* species (Fig. 4.5 & 4.6).

Based on pfbA sequences, we were able to reassign five *Staphylococcus* species back to the *Staphylococcus* genus after they were separated into the *Mammaliicoccus* genus by Madhaiyan et al. (2020) (Fig. 4.7 and 4.8).

Also, we could correct the gene's name from pelL (pectate lyase) and right-handed parallel beta-helix repeat-containing protein [*Staphylococcus muscae*], conserved hypothetical protein, or cell wall surface anchor family protein to plasmin and fibronectin-binding protein A (pfbA) (cell wall surface anchor family protein).

The PCR test presented here offers a low-cost, accurate approach for distinguishing 42 species of the *Staphylococcus* genus, which may be difficult to distinguish based just on morphology. We could also replicate the experiment on other bacterial genera and species by using their *pfbA* gene sequences. However, this would require a large number of additional bacterial genera, species, and isolates to be examined, which was beyond the scope of this study. In this research, we developed a novel molecular technique for the identification, discrimination, and examining the genetic diversity of large, complex microbial communities, for example, *Staphylococcus* species, using conventional and/or quantitative PCR/HRM systems for the identification and discrimination of *Staphylococcus* species. Our *pfbA* gene could be considered as a novel target for specific identification of *Staphylococcus* species and other bacterial species. Wrong identification leads to wrong treatment therefore, the *pfbA* gene is a great genetic marker to reveal the ambiguity in typing bacteria taxonomy, using *Staphylococcus* species as an example.

**Table 4.1.** pfbA genes and genomes of the following bacterial species (762 isolates) used in this study.

Species	Strain	Accession number	Host	Genome status	Citation
<i>S. agnetis</i>	908	CP009623.1	broiler	Finished	Alrubaye et al., 2015
	1379	CP045927.1	bovine	Finished	Shwani et al., 2020
	1387	WMFL000000000.1	bovine	Draft	Shwani et al., 2020
	12B	CP031266.1	buffalo	Finished	None
	1416	WMFQ000000000.1	broiler	Finished	Shwani et al., 2020
	CBMRN_20813338	JPRT01000001.1	bovine	Draft	Calcutt et al., 2014
	1383	WMFO01000000.1	bovine	Draft	Shwani et al., 2020
	1384	WMFN01000000.1	bovine	Draft	Shwani et al., 2020
	1385	WMFM01000000.1	bovine	Draft	Shwani et al., 2020
	1389	WMFK01000000.1	bovine	Draft	Shwani et al., 2020
	1390	WMFJ01000000.1	bovine	Draft	Shwani et al., 2020
	1391	WMFI01000000.1	bovine	Draft	Shwani et al., 2020
	1392	WMFH01000000.1	bovine	Draft	Shwani et al., 2020
	59	MRYT01000009.1	bovine	Draft	None

	NDYM	NDYM01000008.1	broiler	Draft	Poulsen et al., 2017
	NEDS	NEDS01000018.1	broiler	Draft	Poulsen et al., 2017
	NEFX	NEFX01000017.1	broiler	Draft	Poulsen et al., 2017
	SNUC_2265	PZEA01000010.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4805	PZDT01000006.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3610	PZDX01000035.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3261	PZDY01000008.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4051	PZDV01000012.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2493	PZDZ01000005.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1371	PZEC01000009.1	Holstein	Draft	Naushad et al., 2016
	SNUC_725	PZED01000003.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1383	PZEB01000004.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5631	PZDU01000007.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5151	PZDS01000007.1	Holstein	Draft	Naushad et al., 2016
	DSM_23656	PPQF01000004.1	bovine	Draft	None
	3682	VKCY01000009.1	bovine	Draft	None
<i>S. argenteus</i>	58113	AP018562.1	human	Finished	None
	XNO62	CP023076.1	human	Finished	None

	XNO106	CP025023.1	human	Finished	None
	B3-25B	CP042286.1	Holstein	Finished	None
	MSHR1132	FR821777.2	human	Finished	Holt et al., 2011
	PR02	AOHL00000000.1	human	Draft	None
	SARG0275	BHEM00000000.1	NA	Draft	None
	TUM19485	BLRS01000005.1	human	Draft	None
	BN75	CP015758.1	gorilla	Finished	None
	3688STDY6125135	FQMT01000003.1	NA	Finished	None
	3688STDY6125140	FQMZ01000003.1	NA	Finished	None
	3688STDY6125143	FQNB01000003.1	NA	Finished	None
	3688STDY6125069	FQRB01000003.1	NA	Finished	None
	3688STDY6125067	FQRC01000003.1	NA	Finished	None
	3688STDY6125068	FQRD01000003.1	NA	Finished	None
	3688STDY6125089	FQRL01000003.1	NA	Finished	None
	O-10	FXVJ01000090.1	human	Draft	None
	H1864	FXVU01000060.1	human	Draft	None
	M4143	FXVV01000020.1	human	Draft	None
	O-3	FXVW00000000.1	human	Draft	None
	F87619	JGHK01000016.1	human	Finished	None
	M21126	JGMK01000015.1	human	Finished	None
	RK308	LSFQ01000042.1	human	Finished	None
	SJTU_F21224	LWAQ01000009.1	human	Finished	None
	ABFQM	LYLU01000022.1	human	Finished	None
	CCUG_69384	NSBY01000018.1	human	Finished	None
	DSM_28299	PPPZ01000044.1	human	Finished	None
	ST2250	QLNO01000006.1	human	Finished	None
	PHL3431	QGOV01000005.1	human	Finished	None
	PHL3433	QQOW01000006.1	human	Finished	None
<i>S. arlettae</i>	CVD059	ALWK00000000.1	human	Draft	Dinakaran et al., 2012

	NBRC_109765	BKAV00000000.1	poultry	Draft	None
	NCTC12413	UGZE00000000.1	NA	Draft	None
	P2	AP019698.1	floor	Finished	None
	NCTC_12413	PPQB00000000.1	poultry	Draft	None
	SNUC_4786	PZDJ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1715	PZDK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4426	PZDL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4292	PZDM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3447	PZDN00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3029	PZDO00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2101	PZDP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1576	PZDQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1330	PZDR00000000.1	Holstein	Draft	Naushad et al., 2016
	Bari1	QLIZ00000000.1	cabinet	Draft	None
	Bari2	QLJA00000000.1	cabinet	Draft	None
	SNUC_5134	QXRT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4935	QXRU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4202	QXRV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3131	QXRW00000000.1	Holstein	Draft	Naushad et al., 2016



	SNUC_1480	QXRX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1401	QXRY00000000.1	Holstein	Draft	Naushad et al., 2016
	IOV5	SPPT00000000.1	human	Draft	None
	DE0598	VDTT00000000.1	environment	Draft	None
<b><i>S. aureus</i></b>	1510	JACEHY0.1	broiler	Draft	None
	1516	JACEHX0.1	broiler	Draft	None
	JKD6008	CP002120.1	human	Finished	Howden et al., 2010
	Tw20	NC_017331	human	Draft	Holden et al., 2010
	GS426	CP084878.1	human	Finished	None
	MR1	ACZQ01000025.1	human	Draft	Lowder et al., 2009
	RF122	AJ938182.1	cow	Finished	Herron-Olson et al., 2007
	TMUS2134	AP014653.1	human	Finished	Yamaguchi et al., 2015
	Mu50	BA000017.4	human	Finished	Ohta et al., 2004
	N315	BA000018.3	human	Finished	Kuroda et al., 2001
	MW2	BA000033	human	Finished	Baba et al., 2002
	MRSA252	BX571856.1	human	Finished	Holden et al., 2004
	MSSA476	BX571857.1	human	Finished	Holden et al., 2004
	col	CP000046.1	human	Finished	Gill et al., 2005

	NCTC_8325	CP000253	human	Finished	Gillaspy et al., 2006
	USA300_FPR3757	CP000255.1	human	Finished	Diep et al., 2006
	JH9	CP000703	NA	Finished	None
	JH1	CP000736.1	NA	Finished	None
	08BA02176	CP003808.1	human	Finished	Golding et al., 2012
	NCTC_8325	NC_007795.1	human	Draft	Gillaspy et al., 2006
	CN1	CP003979.1	human	Finished	Chen et al., 2013
	KUH140013	AP020311.1	human	Finished	None
	ST228	NC_020568	human	Draft	Vogel et al., 2012
	Mu3	AP009324.1	human	Finished	Hiramatsu et al., 1997
	Newman	AP009351.1	human	Finished	Baba et al., 2008
	04-02981	NC_017340	human	Draft	Nübel et al., 2010
	ATCC_25923	CP009361.1	human	Finished	Treangen et al., 2014
	ED98	CP001781.1	human	Finished	Lowder et al., 2009
	JH1	NC_009632.1	human	Draft	None
	N315	NC_002745.2	human	Draft	Kuroda et al., 2001
<i>S. auricularis</i>	NCTC_12101	PPQW00000000.1	human	Draft	None
	DSM_20609	LLER0000000.1	human	Draft	None
	NCTC_12101	LS483491.1	human	Finished	None

	SNUC_3034	PZDH00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_993	PZDI00000000.1	Holstein	Draft	Naushad et al., 2016
	DE0381	VTQJ00000000.1	environment	Draft	None
<i>S. capitis</i>	SK14	ACFR00000000.1	human	Draft	None
	C87	ACRH00000000.1	human	Draft	<b>None</b>
	VCU116	AFTX00000000.1	human	Draft	None
	QN1	AJTH00000000.1	human	Draft	None
	CR01	CBUB00000000.1	human	Draft	None
	CR07	CZWH00000000.1	human	Draft	None
	CR02	CZWI00000000.1	human	Draft	None
	LNZR-1	JGYJ00000000.1	human	Draft	None
	AYP1020	CP007601.1	human	Finished	Cameron et al. , 2015
	FDAARGOS_378	CP023966.1	human	Finished	None
	FDAARGOS_753	CP053957.1	human	Finished	None
	TW2795	AP014956.1	human	Finished	None
	NCTC_11045	PPPY00000000.1	human	Draft	None
	DSM_6717	PPQI00000000.1	human	Draft	None
	SNUC_6079	PZCT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5871	PZCU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4705	PZCV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4231	PZCX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3769	PZCY00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3379	PZCZ00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_2974	PZDA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2159	PZDD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_895	PZDF00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1642	QXRQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_807	QXRS00000000.1	Holstein	Draft	Naushad et al., 2016
	C2784	RCTS00000000.1	human	Draft	None
	TCR-3	RIYT00000000.1	human	Draft	None
	DE0445	VDSA00000000.1	environment	Draft	None
	DE0440	VDSE00000000.1	environment	Draft	None
	DE0241	VECR00000000.1	environment	Draft	None
<b><i>S. caprae</i></b>	26D	CP031271.1	buffalo	Finished	None
	JMUB145	AP018585	human	Finished	None
	JMUB590	AP018586.1	human	Finished	None
	JMUB898	AP018587	human	Finished	None
	9557	JXXP00000000.1	human	Finished	Zheng et al., 2015
	NCTC_12196	PPRT00000000.1	bovine	Draft	None
	SNUC_4023	QXRK00000000.1	Holstein	Draft	Naushad et al., 2016
	SY333	CP051643.1	human	Finished	None
	NCTC12196	UHCW00000000.1	human	Draft	None
<b><i>S. carnosus</i></b>	NBRC_109623	BKAP00000000.1	NA	Draft	None
	LTH7013	LAIU00000000.1	ham	Draft	Müller et al., 2015
	336	LISV00000000.1	beef	Draft	None

	LTH_3730	CP016760.1	Pla-chom fish	Finished	None
	DSM_11676	PPRE00000000.1	fish sauce	Draft	None
	TM300	AM295250.1	NA	Finished	Rosenstein et al., 2009
	NCTC_13825	UHCT00000000.1	NA	Draft	None
	NCTC_13826	UHCY00000000.1	NA	Draft	None
	NBRC_109622	BKAO00000000.1	NA	Draft	None
<i>S. chromogenes</i>	MU970	JMJF00000000.1	bovine	Draft	Fry et al., 2014
	92	MRYX00000000.1	bovine	Draft	None
	101	MRYX00000000.1	bovine	Draft	None
	121	MRYZ00000000.1	bovine	Draft	None
	117	MRZA00000000.1	bovine	Draft	None
	38	MRZB00000000.1	bovine	Draft	None
	1401	CP046028.1	broiler	Draft	Shwani et al., 2020
	17A	CP031274.1	buffalo	Draft	None
	20B	CP031471.1	buffalo	Draft	None
	34B	CP031470.1	buffalo	Draft	None
	46	MRYW00000000.1	bovine	Draft	None
	NCTC_10530	PPQK00000000.1	bovine	Draft	None
	SNUC_4199	PYZV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4042	PYZX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2579	PZAC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2487	PZAD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1405	PZAK00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_5084	PZBK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1063	PZBS00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_277	PZCA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_265	PZCB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_134	PZCE00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_133	PZCF00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_107	PZCL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_105	PZCM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_91	PZCO00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1508	QXQY00000000.1	Holstein	Draft	Naushad et al., 2016
	S48	SUMU00000000.1	cow	Draft	None
	NCTC_10530	UHDB00000000.1	NA	Draft	None
	SDA1	VJNG00000000.1	desert soil	Draft	None
<b><i>S. cohnii</i></b>	hu-01	AYOS00000000.1	human	Draft	None
	NBRC_109713	BKAS01000014.1	human	Finished	None
	SE4.1	JRVV00000000.1	rice seed	Draft	None
	SE4.2	JRVW00000000.1	rice seed	Draft	None
	SE4.4	JRVY00000000.1	rice seed	Draft	None
	G22B2	LAKJ00000000.1	human	Draft	None
	57	LATU00000000.1	human	Draft	None
	MF1844	LSKX00000000.1	poultry eqp	Draft	Fagerlund et al., 2016

	SW120	MPPU00000000.1	wolf	Draft	None
	ATCC_29974	LT963440.1	NA	Draft	None
	FDAARGOS_334	CP027422.1	human	Finished	None
	FDAARGOS_538	CP033735.1	human	Finished	None
	FDAARGOS_744	CP054807.1	human	Finished	None
	SNUDS-2	CP019597.1	duck	Finished	None
	NCTC_11041	PPQC00000000.1	human	Draft	None
	DSM_6718	PPRL00000000.1	human	Draft	None
	SNUC_5656	PYZO00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3829	PYZR00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_156	PZBD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4643	QXSX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1067	QXTC00000000.1	Holstein	Draft	Naushad et al., 2016
	YNSA55	VCFA00000000.1	human	Draft	None
	DE0524	VDPN00000000.1	environment	Draft	None
	DE0450	VDRW00000000.1	environment	Draft	None
	DE0431	VDSK00000000.1	environment	Draft	None
	DE0360	VDZN00000000.1	environment	Draft	None
	DE0303	VEBD00000000.1	environment	Draft	None
	DE0122	VEFY00000000.1	environment	Draft	None
	DE0550	VTQD00000000.1	environment	Draft	None
<i>S. condimenti</i>	DSM_11674	LAQN00000000.1	soy sauce mash	Draft	Zheng et al., 2016
	DSM11674	CP015114.1	soy sauce mash	Draft	None

	NCTC_13827	LR134360.1	soy sauce mash	Draft	None
	StO_2014-01	CP018776.1	human	Finished	None
	DSM_11674	PPQY00000000.1	soy sauce mash	Draft	None
	SA11	RQTE00000000.1	human	Draft	None
	SAM1	RQTG00000000.1	animal	Draft	None
	DE0480	VDQW00000000.1	environment	Draft	None
<i>S. delphini</i>	8086	CAIA00000000.1	animal	Draft	None
	14S03309-1	MWRM00000000.1	pigeon	Draft	Verstappen et al., 2017
	14S03311-1	MWRN00000000.1	horse	Draft	Verstappen et al., 2017
	14S03313-1	MWRO00000000.1	horse	Draft	Verstappen et al., 2017
	14S03314-1	MWRP00000000.1	pigeon	Draft	Verstappen et al., 2017
	14S00091-1	MWUN00000000.1	dolphin	Draft	Verstappen et al., 2017
	14S02207-1	MWUO00000000.1	horse	Draft	Verstappen et al., 2017
	14S03318-1	MWUP00000000.1	marter	Draft	Verstappen et al., 2017
	14S03319-1	MWUQ00000000.1	horse	Draft	Verstappen et al., 2017
	15S02591-1	MWUR00000000.1	dolphin	Draft	Verstappen et al., 2017
	215102607201-2	MWUS00000000.1	horse	Draft	Verstappen et al., 2017
	215100905101-2	MWUT00000000.1	horse	Draft	Verstappen et al., 2017



	215070706401-1	MWUU00000000.1	horse	Draft	Verstappen et al., 2017
	215062304401-1	MWUV00000000.1	horse	Draft	Verstappen et al., 2017
	214092504301-1	MWUW00000000.1	horse	Draft	Verstappen et al., 2017
	Heidy	NIPK00000000.1	purulent material	Draft	Verstappen et al., 2017
	215100905101-2	MWUT00000000.1	horse	Draft	Verstappen et al., 2017
	NCTC_12225	LR134263.1	human skin	Finished	None
	NCTC_12225	PPRV00000000.1	purulent material	Draft	None
	BCW_7426	SDSN00000000.1	whale	Draft	None
	P5747	WNLD00000000.1	penguin	Draft	Vrbovska et al., 2020
	P6456	WNLE00000000.1	penguin	Draft	Vrbovska et al., 2020
<i>S. devriesei</i>	CCUG_58238	PPRG00000000.1	cow	Draft	None
	SNUC_4143	PYZH00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1409	PYZI00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1316	PYZJ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1156	PYZK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_761	PYZL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_593	PYZM00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_4438	QXSU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_760	QXSV00000000.1	Holstein	Draft	Naushad et al., 2016
	NCTC_13828	UHCZ00000000.1	NA	Draft	None
<i>S. epidermidis</i>	949_S8	CP010942.1	human	Finished	Biswas et al., 2015
	AMT	CP022247.1	human	Finished	MacLea et al., 2020
	CSF41498	CP030246.1	human	Finished	Galac et al., 2019
	CDC120	CP034111.1	human	Draft	None
	ATCC_14990	CP035288.1	human	Finished	None
	E73	CP035643.1	human	Finished	None
	HD33	CP040864.1	human	Finished	None
	HD66	CP040868.1	human	Finished	None
	SESURV_p1_0557	CP043777.1	human	Draft	Zhou et al., 2020
	SESURV_p1_0563	CP043781.1	human	Draft	Zhou et al., 2020
	SESURV_p3_0825	CP043792.1	human	Draft	Zhou et al., 2020
	SESURV_p1_1200	CP043796.1	human	Draft	Zhou et al., 2020
	SESURV_p3_1362	CP043801.1	human	Draft	Zhou et al., 2020
	NCCP_16829	CP043841.1	human	Finished	None
	ATCC_12228	CP043845.1	human	Finished	None
	IRL01	CP045648.1	human	Finished	None
	NCTC_13924	LR134536.1	human	Finished	None
	none	LR735421.1	human	Draft	None
	none	LR735429.1	human	Draft	None

	none	LR735432.1	human	Draft	None
	none	LR735434.1	human	Draft	None
	none	LR735437.1	human	Draft	None
	none	LR735440.1	human	Draft	None
	ATCC_12228	NC_004461.1	human	Finished	Zhang et al., 2003
	SEI	CP009046.1	human	Finished	Davenport et al., 2014
	ATCC_12228	AE015929.1	human	Draft	None
	CDC121	CP034115.1	human	Draft	None
	NBRC_100911	AP019721.1	human	Finished	None
	RP62A	CP000029.1	human	Finished	Gill et al., 2005
	SESURV_p4_1553	CP043804.1	human	Draft	Zhou et al., 2020
<b><i>S. equorum</i></b>	UMC-CNS-924	AVBD00000000.1	cow	Draft	Calcutt et al., 2013
	Mu2	CAJL00000000.1	cow	Draft	none
	G8HB1	LAKE00000000.1	cow	Draft	none
	900_4	LNDI00000000.1	cheese rind	Draft	none
	862_5	LNMY00000000.1	cheese rind	Draft	none
	White_SAM	LNMZ00000000.1	cheese rind	Draft	none
	OffWhite_SAM	LNNA00000000.1	cheese rind	Draft	none
	BC9	LNNB00000000.1	cheese rind	Draft	none
	BC3	LNNC00000000.1	cheese rind	Draft	none
	341_10	LNND00000000.1	cheese rind	Draft	none
	738_7	LNPX00000000.1	cheese rind	Draft	none
	908_10	LNPY00000000.1	cheese rind	Draft	none
	947_12	LNPZ00000000.1	cheese rind	Draft	none
	RE2.24	LWJS00000000.1	rice seed	Draft	none
	RE2.35	LWJU00000000.1	rice seed	Draft	none

	RE2.40	LWJW00000000.1	rice seed	Draft	none
	AR8-13	MDJP00000000.1	Anopheles	Draft	none
	876_5	NMOI00000000.1	cheese rind	Draft	none
	82b3	CP041697.1	dust sample	Finished	none
	C2014	CP013714.1	salted food	Finished	none
	KM1031	CP013980.1	salted food	Finished	none
	KS1039	CP013114.1	salted food	Finished	none
	DSM_15097	PPQL00000000.1	cheese rind	Draft	none
	SNUC_5474	PYYG00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1644	PYYK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_476	PYYM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_193	PYYN00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2835	QXSF00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1023	QXSH00000000.1	Holstein	Draft	Naushad et al., 2016
	NCTC12414	UHDI00000000.1	cow	Draft	none
<i>S. felis</i>	ATCC_49168	CP027770.1	cat	Finished	none
	DSM_7377	PPRI00000000.1	cat	Draft	none
	F1	QKXJ00000000.1	cat	Draft	Worthing et al., 2018
	F2	QKXK00000000.1	cat	Draft	Worthing et al., 2018
	F4	QKXL00000000.1	cat	Draft	Worthing et al., 2018
	F5	QKXM00000000.1	cat	Draft	Worthing et al., 2018

	F6	QKXN000000000.1	cat	Draft	Worthing et al., 2018
	F7	QKXO000000000.1	cat	Draft	Worthing et al., 2018
	F8	QKXP000000000.1	cat	Draft	Worthing et al., 2018
	F9	QKXQ000000000.1	cat	Draft	Worthing et al., 2018
	F12	QKXR000000000.1	cat	Draft	Worthing et al., 2018
	F13	QKXS000000000.1	cat	Draft	Worthing et al., 2018
	F14	QKXT000000000.1	cat	Draft	Worthing et al., 2018
	F15	QKXU000000000.1	cat	Draft	Worthing et al., 2018
	F16	QKXV000000000.1	cat	Draft	Worthing et al., 2018
	F17	QKXW000000000.1	cat	Draft	Worthing et al., 2018
	F18	QKXX000000000.1	cat	Draft	Worthing et al., 2018
	F19	QKXY000000000.1	cat	Draft	Worthing et al., 2018
	F20	QKXZ000000000.1	cat	Draft	Worthing et al., 2018
	F21	QKYA000000000.1	cat	Draft	Worthing et al., 2018
	F22	QKYB000000000.1	cat	Draft	Worthing et al., 2018
	F24	QKYC000000000.1	cat	Draft	Worthing et al., 2018

	F25	QKYD00000000.1	cat	Draft	Worthing et al., 2018
	F26	QKYE00000000.1	cat	Draft	Worthing et al., 2018
	F27	QKYF00000000.1	cat	Draft	Worthing et al., 2018
	F29	QKYG00000000.1	cat	Draft	Worthing et al., 2018
	F30	QKYH00000000.1	cat	Draft	Worthing et al., 2018
	F33	QKYI00000000.1	cat	Draft	Worthing et al., 2018
	F34	QKYJ00000000.1	cat	Draft	Worthing et al., 2018
<b><i>S. fleurettii</i></b>	FDAARGOS_682	CP046351.1	Jugular catheter	Finished	none
	NCTC_12218	RXXB00000000.1	Jugular catheter	Draft	none
	ssch2	CP064058.1	human	Finished	none
	ssch3	CP064059.1	human	Finished	none
<b><i>S. gallinarum</i></b>	NBRC_109767	BKAX00000000.1	NA	Draft	none
	DSM_20610	JXCF00000000.1	chicken skn	Draft	none
–	SNUC_1244	PYYC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_395	PYYD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4861	PZIZ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5382	PZJA00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_4236	PZJB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4089	PZJC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2111	PZJD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2087	PZJE00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1388	QXRZ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1169	QXSA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1046	QXSB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_741	QXSC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_302	QXSD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5344	QXVL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1606	QXVM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4633	QYJO00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4583	QYJP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3067	QYJQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2913	QYJR00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2094	QYJS00000000.1	Holstein	Draft	Naushad et al., 2016
	NCTC_12195	UHDK00000000.1	NA	Draft	none

<i>S. haemolyticus</i>	JCSC1435	AP006716.1	NA	Finished	Takeuchi et al., 2005
	S167	CP013911.1	leaf vegetable	Finished	Hong et al 2016
	83131A	CP024809.1	human	Finished	None
	SGAir0252	CP025031.1	air	Finished	Premkrishnan et al 2018
	83131B	CP025396.1	human	Finished	None
	FDAARGOS_517	CP033814.1	human	Finished	None
	URN1-2019	CP052055.1	human	Finished	None
	PHRX1-2019	CP052056.1	human	Finished	None
	51-33	CUDQ00000000.1	human	Draft	None
	51-37	CUDV00000000.1	human	Draft	None
	CN1138	CUEV00000000.1	NA	Draft	None
	8074328	CUFG00000000.1	NA	Draft	None
	134634	CUGS00000000.1	Human CSF	Draft	None
	140376	CUHF00000000.1	human	Draft	None
	105731	CUHI00000000.1	catheter	Draft	None
	7532	CUHM00000000.1	human	Draft	None
	235_SHAE	JVPA00000000.1	human	Draft	Roach et al 2015
	ATCC_29970	CP035291.1	human	Finished	None
	K8	LT963441.1	NA	Draft	None
	PK-01	CP035541.1	human	Finished	None
	Sh29	CP011116.1	human	Finished	Almeida et al 2015
	G811N2B1	PGWX00000000.1	human	Draft	None
	A109N1B1	PGWY00000000.1	human	Draft	None
	0878	QVPW00000000.1	human	Draft	None
	0894	QVPX00000000.1	human	Draft	None
	MDMC083	RXGD00000000.1	desert sand	Draft	None



	SDB1	VJMO00000000.1	desert soil	Draft	None
<b><i>S. hominis</i></b>	C80	ACRM00000000.1	human	Draft	None
	ZBW5	AKGC00000000.1	human	Draft	Jiang et al 2012
	FDAARGOS_136	CP014107.1	human	Finished	None
	FDAARGOS_575	CP033732.1	human	Finished	None
	FDAARGOS_747	CP046301.1	human	Finished	None
	FDAARGOS_745	CP050982.1	human	Finished	None
	FDAARGOS_762	CP054006.1	human	Finished	None
	FDAARGOS_661	CP054550.1	human	Finished	None
	FDAARGOS_748	CP054883.1	human	Finished	None
	J11	FBVJ00000000.1	human	Draft	None
	J31	FBVO00000000.1	human	Draft	None
	MMP2	LNTW00000000.1	Mammoth	Draft	None
	HMSC034C12	LTRZ00000000.1	human	Draft	None
	RE2.9	LWJQ00000000.1	rice seed	Draft	None
	LRKNS031	LXRS00000000.1	human	Draft	None
	Hudgins	MAYR00000000.1	human	Draft	None
	BHG17	MPNR00000000.1	goose	Draft	None
	19A	CP031277.1	buffalo	Finished	None
	FDAARGOS_746	CP046306.1	human	Finished	None
	K1	CP020618.1	human	Finished	None
	J11	LT963438.1	human	Draft	None
	J6	LT963442.1	human	Draft	None
	SH04_17	PHKJ00000000.1	human	Draft	None
	SH08_17	PHKL00000000.1	human	Draft	None
	NCTC_11320	PPQE00000000.1	human	Draft	None
	CCUG_42399	PPQX00000000.1	human	Draft	None
	SNUC_2694	PZHZ00000000.1	Holstein	Draft	Naushad et al., 2016
	APC_3824	SHFC00000000.1	human	Draft	None

	SDA3	VJML00000000.1	desert soil	Draft	None
	SHYJILJH	WJUO00000000.1	soil	Draft	None
<b><i>S. hyicus</i></b>	NCTC_10350	LS483304.1	NA	Finished	None
	1381	WMFG00000002.1	bovine	Draft	Shwani et al., 2020
	1388	WMFF00000002.1	bovine	Draft	Shwani et al., 2020
	ATCC_11249	CP008747.1	bovine	Draft	Calcutt et al., 2015
	SNUC_4992	PZHT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5426	PZHU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5959	QXVO00000000.1	Holstein	Draft	Naushad et al., 2016
	1380	WMFP00000000.1	bovine	Draft	Shwani et al., 2020
<b><i>S. kloosii</i></b>	ATCC_43959	CP027846.1	squirrel	Finished	None
	NBRC_109624	PPQQ00000000.1	squirrel	Draft	None
	NCTC_12415	PPQQ00000000.1	squirrel	Draft	None
	SNUC_4696	PZHS00000000.1	Holstein	Draft	Naushad et al., 2016
	NCTC_12415	UHDQ00000000.1	NA	Draft	None
<b><i>S. lentus</i></b>	F1142	AJXO00000000.1	doenjang	Draft	Nam et al., 2012
	050AP	FMRW00000000.1	human	Draft	None
	MF1767	LSKV00000000.1	poultry eqp	Draft	Fagerlund et al., 2016
	MF1862	LSKY00000000.1	poultry eqp	Draft	Fagerlund et al., 2016

	H29	CP059679.1	chickens	Draft	None
	NCTC_12102	PPRS00000000.1	goat	Draft	None
	HT5	SPOY00000000.1	oral swab	Draft	None
	AE2	SPPP00000000.1	oral swab	Draft	None
	NCTC_12102	UHDR00000000.1	NA	Draft	None
<b><i>S. lugdunensis</i></b>	ACS-027-V-Sch2	AGZW00000000.1	NA	Draft	None
	FDAARGOS_141	CP014022.1	human	Finished	None
	Klug93G-4	CP017069.2	human	Finished	Ho et al., 2016
	FDAARGOS_222	CP020406.2	human	Finished	None
	VISLISI_27	CP020735.1	human	Finished	Argemi et al., 2017
	VISLISI_21	CP020762.1			
	VISLISI_25	CP020763.1	human	Finished	Argemi et al., 2017
	VISLISI_22	CP020764.1	human	Finished	None
	VISLISI_33	CP020769.1	human	Finished	None
	FDAARGOS_381	CP023970.1	human	Finished	None
	APC_3758	CP038807.1	human	Finished	None
	SL13	CP041722.1	human	Finished	None
	SL29	CP041723.1	human	Finished	None
	SL55	CP041724.1	human	Finished	None
	SL117	CP041725.1	human	Finished	None
	SL118	CP041726.1	human	Finished	None
	SL122	CP041727.1	human	Finished	None
	VCU148	JIBR00000000.1	human	Draft	None
	VCU150	JIBS00000000.1	human	Draft	None
	MJR7738	LRQI00000000.1	human	Draft	None
	NCTC7990	LS483312.1	human	Finished	None
	NCTC12217	LS483482.1	human	Finished	None
	FDAARGOS_143	NZ_CP014023.1	human	Draft	None

	FDAARGOS_377	CP023539.1	human	Finished	None
	HKU09-01	CP001837.1	human	Finished	Tse et al., 2010
	JICS135	AP021848.1	human	Finished	None
	N920143	NC_017353.1	human	Draft	None
	NCTC_12217	PPPV000000000.1	human	Draft	None
	E7	SCHB000000000.1	human	Draft	None
<i>S. lutrae</i>	ATCC700373	CP020773.1	otter	Finished	None
	DSM_10244	PPRH000000000.1	otter	Draft	None
<i>S. muscae</i>	NCTC_13833	LT906464.1	stable fly	Finished	None
	ATCC_49910	CP027848.1	flies	Finished	None
	DSM_7068	PPQJ000000000.1	stable fly	Draft	None
<i>S. nepalensis</i>	JS1	CP017460.1	fermented food	Finished	None
	JS11	CP017466.1	fermented food	Finished	None
	JS9	CP017459.1	fermented food	Finished	None
	DSM_15150	PPRR000000000.1	goat	Draft	None
	SNUC_4337	PZHR000000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4025	QXVN000000000.1	Holstein	Draft	Naushad et al., 2016
	NCTC_13834	UHDS000000000.1	NA	Draft	None
<i>S. pasteurii</i>	NFIX07	FPKT000000000.1	human	Draft	None
	BAB3	LAKF000000000.1	human	Draft	None
	PT#26	LXWH000000000.1	wild swine		

	SNUC_5836	QAVY00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5329	QAVZ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2044	QAWA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2657	QXVV00000000.1	Holstein	Draft	Naushad et al., 2016
	RIT605	RJLY00000000.1	mobile phone	Draft	None
	DE0522	VDPP00000000.1	environment	Draft	None
	DE0452	VDRU00000000.1	environment	Draft	None
	DE0353	VDZT00000000.1	environment	Draft	None
	DE0376	VEJR00000000.1	environment	Draft	None
	DE0437	VTQG00000000.1	environment	Draft	None
	3C	CP031280.1	human	Finished	None
	JS7	CP017463.1	human	Finished	None
<b><i>S. pettenkoferi</i></b>	FDAARGOS_288	CP022096.2	human	Finished	None
	UGA20	NWTY00000000.1	human	Draft	None
	UMB0834	PNGG00000000.1	human	Draft	None
	CCUG_51270	PPRN00000000.1	human	Draft	None
	VCU012	AGUA00000000.1	NA	Draft	None
	DE0528	VDPJ00000000.1	environment	Draft	None
	DE0475	VDRA00000000.1	environment	Draft	None
	DE0465	VDRK00000000.1	environment	Draft	None
	DE0267	VEBZ00000000.1	environment	Draft	None
	DE0227	VECY00000000.1	environment	Draft	None
	DE0160	VEEV00000000.1	environment	Draft	None
	DE0158	VEEX00000000.1	environment	Draft	None
	DE0155	VEEY00000000.1	environment	Draft	None
	DE0149	VEFD00000000.1	environment	Draft	None

	DE0147	VEFF00000000.1	environment	Draft	None
	DE0138	VEFM00000000.1	environment	Draft	None
<i>S. piscifermentans</i>	NCTC_13836	LT906447.1	shrimp	Finished	None
	NBRC_109625	BKAR00000000.1	shrimp	Draft	None
<i>S. pseudintermedius</i>	49_44	CP035743.1	dog	Finished	None
	5912	CP009120.1	dog	Finished	None
	081661	CP016073.1	dog	Finished	Riely et al., 2016
	SP79	AP019372.1	dog	Finished	None
	53_88	CP035740.1	human	Finished	None
	53_60	CP035741.1	human	Finished	None
	51_92	CP035742.1	dog	Finished	None
	157588	CP054206.2	dog	Finished	None
	ED99	NC_017568.1	dog	Finished	Zakour et al., 2011
	G3C4	CP032682.1	dog	Finished	None
	HKU10-03	NC_014925.1	dog	Draft	None
	E104	LAWU00000000.1	dog	Draft	None
	SL/085	MQNB00000000.1	dog	Draft	Verstappen et al., 2017
	SL/154	MQNF00000000.1	dog	Draft	Verstappen et al., 2017
	NA45	NZ_CP016072.1	dog	Draft	Riely et al., 2016
	2080722072011	PEOJ00000000.1	dog	Draft	Verstappen et al., 2017
	738	PHHZ00000000.1	instrument	Draft	None
	684	PHIA00000000.1	dog	Draft	None

	114N	PJUQ00000000.1	dog	Draft	Abouelkhair et al., 2018
	MAD417	QGOQ00000000.1	dog	Draft	None
	MAD429	QGOV00000000.1	human	Draft	None
	VTH625	QGPC00000000.1	dog	Draft	None
	MAD400	QGPL00000000.1	dog	Draft	None
	SP128a	QHIE00000000.1	dog	Draft	Nisa et al., 2019
	MI_14-3131	QMHS00000000.1	horse	Draft	None
	MI_12-1817	QMHT00000000.1	horse	Draft	None
	MI_07-1650	QMHU00000000.1	horse	Draft	None
<i>S. saprophyticus</i>	1A	CP031196.1	human	Finished	None
	883	LT963436.1	human	Draft	None
	KACC_16562	AHKB00000000.1	fish	Draft	Kim et al., 2012
	ATCC_15305	CP035294.1	human	Finished	None
	ATCC_15306	AP008934.1	human	Draft	Kuroda et al., 2005
	FDAARGOS_336	CP022056.2	human	Finished	None
	FDAARGOS_355	CP022093.2	human	Finished	None
	UTI-035	CP054434.1	human	Finished	None
	UTI-042y	CP054438.1	human	Finished	None
	UTI-058y	CP054440.1	human	Finished	None
	UTI-056	CP054444.1	human	Finished	None
	UTI-050	CP054575.1	human	Finished	None
	UTI-045	CP054831.1	human	Finished	None
	UBA2710	DEKY00000000.1	human	Draft	Parks et al., 2017
	UBA5034	DICE00000000.1	metal	Draft	Parks et al., 2017

	UBA5645	DIJL00000000.1	metal	Draft	Parks et al., 2017
	UBA6634	DKLS00000000.1	metal	Draft	Parks et al., 2017
	7108	LMYQ00000000.1	human	Draft	None
	1146	LMZL00000000.1	human	Draft	None
	3201	LMZM00000000.1	human	Draft	None
	429A	LNPK00000000.1	cheese rind	Draft	None
	MF6029	LSLC00000000.1	poultry eqp	Draft	Fagerlund et al., 2016
	DPC5671	MUXI00000000.1	cheese	Draft	None
	ATCC_15307	NC_007350.1	human	Draft	Kuroda et al., 2005
	SNUC 2373	QXVJ00000000.1	Holstein	Draft	Naushad et al., 2016
	AG1	SAYP00000000.1	soil	Draft	None
	SS410	SDLY00000000.1	human	Draft	None
<i>S. schleiferi</i>	1360-13	NZ_CP009470.1	dog	Draft	Misic et al., 2015
	2317-03	CP010309.1	dog	Finished	Misic et al., 2015
	5909-02	CP009676.1	dog	Finished	Misic et al., 2015
	2142-05	NZ_CP009762.1	dog	Draft	Misic et al., 2015
	OT1-1	CP035007.1	human	Draft	None
	196	POVH00000000.1	human	Draft	None
	DSM_6628	PPQN00000000.1	dog	Draft	None
	TSCC54	AP014944.1	dog	Finished	Sasaki et al., 2015
	1028910	VFBN00000000.1	dog	Draft	None



<i>S. schweitzeri</i>	NCTC_13712	LR134304.1	NA	Finished	None
	26	CCEG00000000.1	NA	Draft	None
	54	CCEO00000000.1	NA	Draft	None
	38	CCEQ00000000.1	NA	Draft	None
	52	PPQS00000000.1	monkey	Draft	None
<i>S. sciuri</i>	NCTC_12103	LS483305.1	NA	Finished	None
	FDAARGOS_285	CP022046.2	human	Finished	None
	B9-58B	CP041879.1	human	Draft	None
	Z8	JANE00000000.1	human	Draft	Hu et al., 2015
	NS1	LDTK00000000.1	Asian rice	Draft	Midha et al., 2016
	NS53	LDTP00000000.1	Asian rice	Draft	Midha et al., 2016
	RSA37	LDTQ00000000.1	Asian rice	Draft	Midha et al., 2016
	SAP15-1	MAXU00000000.1	pig	Draft	None
	P575	MDVU00000000.1	human	Draft	Zeman et al., 2017
	LCHXa	NADO00000000.1	NA	Draft	None
	NWAF26	CP048732.1	pig	Draft	None
	i1	NXFX00000000.1	African spider	Draft	None
	NCTC_12103	PPQD00000000.1	squirrel	Draft	None
	MC10_S56	PWAC00000000.1	bovine	Draft	None
	SNUC_5594	PZGV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1760	PZHA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1372	PZHC00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_740	PZHE00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2936	QXVA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1679	QXVB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1516	QXVC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_174	QXVD00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3363	QYIW00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1352	QYJB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1345	QYJC00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1043	QYJF00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUDS-18	CP020377.1	duckling	Finished	Han et al., 2013
	GDM7P051A	WIVK00000000.1	swine	Draft	None
	Y98P	WJGI01000005.1	dog	Finished	None
<i>S. simiae</i>	NCTC_13838	LT906460.1	squirrel monkey	Finished	None
	CCM_7213	AEUN01000488.1	squirrel monkey	Finished	Suzuki et al., 2012
	CCUG_51256	PPQR01000028	squirrel monkey	Finished	None
<i>S. simulans</i>	ATCC_27848	LT963435.1	NA	Draft	None
	UMC-CNS-990	AXDY00000000.1	cow	Draft	Calcutt et al., 2013

	CJ16	LJSL00000000.1	human	Draft	Chen & Fang, 2016
	MR1	CP015642.1	sheep	Finished	None
	MR2	CP016157.1	sheep	Finished	None
	MR3	CP017428.1	sheep	Finished	Zarate et al., 2017
	MR4	CP017430.1	sheep	Finished	Zarate et al., 2017
	102	MRZE00000000.1	bovine	Draft	None
	19	MRZL00000000.1	bovine	Draft	None
	NCTC_11046	PPRU00000000.1	human	Draft	None
	SNUC_5405	PZFV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2756	PZFY00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2478	PZFZ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2167	PZGB00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1354	PZGK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1337	PZGL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1336	PZGM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1050	PZGP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_983	PZGQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_67	PZGT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_51	PZGU00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_1392	QXTW00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1347	QXTX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3896	QXUQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1690	QXUT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1325	QXUU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_822	QXUW00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_317	QXUX00000000.1	Holstein	Draft	Naushad et al., 2016
<b><i>S. stepanovicii</i></b>	NCTC_13839	_LT906462.1	Bank vole	Finished	None
	DSM_26319	PPQZ00000000.1	Bank vole	Draft	None
	CCM_7717	BMDM00000000.1	Bank vole	Draft	None
<b><i>S. succinus</i></b>	14BME20	CP018199.1	doenjang	Finished	None
	DSM_14617	LCSH00000000.1	plant_soil	Draft	None
	BC15	LNPF00000000.1	cheese rind	Draft	None
	CSM-77	LUJH00000000.1	halite	Draft	Megaw & Gilmore 2016
	INIFAP_002-15	PIZQ00000000.1	Asian tick	Draft	None
	DSM_14617	PPQP00000000.1	plant_soil	Draft	None
	DSM_15096	PPQV00000000.1	cheese	Draft	None
	SNUC_4645	PZFM00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4324	PZFN00000000.1	Holstein	Draft	Naushad et al., 2016

	SNUC_1916	PZFO00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1280	PZFP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1231	PZFQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1084	PZFR00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_727	PZFS00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_201	PZFT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_6028	QXTP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5955	QXTQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5353	QXTR00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4691	QXTS00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2261	QXTT00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1239	QXTU00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1544	QYIV00000000.1	Holstein	Draft	Naushad et al., 2016
<i>S. vitulinus</i>	FDAARGOS_1153	CP068061.1	human	Finished	None
	FDAARGOS_1207	CP069486.1	ground lamb	Finished	None
	Ani_LG-101	CP050459.1	milk	Finished	None
	Tienloo1	CP051882.1	ground beef	Finished	None

	F1028	AJTR00000000.1	frmntd soybean	Draft	Nam et al., 2012
	DSM_15615	PPRQ00000000.1	ground lamb	Draft	None
	DSM_9930	RXWX00000000.1	human	Draft	None
	FME39	JABUYR00000000.1	cheese rind	Draft	None
	CCM4481	BMDF00000000.1	NA	Draft	None
	SNUC_2204	PZFK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2436	PZFG01000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2780	PZFF01000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_430	QXTO01000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_4525	QXTN00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_730	PZFL00000000.1	Holstein	Draft	Naushad et al., 2016
<b><i>S. warneri</i></b>	16A	CP031269.1	buffalo	Finished	None
	22.1	CP032159.1	lab	Finished	None
	FDAARGOS_754	CP054017.1	human	Finished	None
	SWO	CP033098.1	cndntn water	Draft	None
	WB224	CP053477.1	doubanjiang	Finished	None
	NGS-ED-1001	JPOW00000000.1	human	Draft	Kropp et al., 2014
	691_SWAR	JUWX00000000.1	human	Draft	Roach et al., 2015
	1DB1	LAKH00000000.1	human	Draft	None
	SA9	LDTT00000000.1	Asian rice	Draft	Midha et al., 2016

	NCTC_11044	LR134269.1	human	Draft	None
	NCTC_7291	LR134244.1	human	Finished	None
	UGA28	NWUA00000000.1	human	Draft	None
	UGA3	NWUB00000000.1	human	Draft	None
	TRPF4	PJLY00000000.1	Marine water	Draft	None
	NCTC_11044	PPPW00000000.1	human	Finished	None
	SNUC_2993	PZEV00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2313	PZEX00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_2135	PZYZ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1678	PZFA00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3575	PZFI00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3412	PZFJ00000000.1	Holstein	Draft	Naushad et al., 2016
	YZ-1	QHJX00000000.1	high salinity	Draft	None
	OM08-17AT	QSTD00000000.1	human	Draft	None
	SNUC_4527	QXUO00000000.1	Holstein	Draft	Naushad et al., 2016
	1XD21-27	QXWP00000000.1	mouse	Draft	None
	C2796	RCTT00000000.1	human	Draft	None
	SG1	CP003668.1	lab	Finished	Chen et al., 2013
	DE0562	VDOJ00000000.1	environment	Draft	None
	DE0520	VDPQ00000000.1	environment	Draft	None
	DE0513	VDPX00000000.1	environment	Draft	None
<i>S. xylosus</i>	2	CP031275.1	human	Finished	None
	NJ	ANMR00000000.1	human	Draft	None

	ATCC_29971	LT963439.1	human	Draft	None
	DMB3-Bh1	AURW00000000.1	muse	Draft	None
	C2a	LN554884.1	human	Finished	None
	S170	CP013922.1	leaf	Finished	None
	DMSX03	CP060271.1	meju	Finished	None
	47-83	FMRS00000000.1	Bulk milk	Draft	None
	LSR_02N	JXAU00000000.1	water	Draft	None
	NS341	LDTU00000000.1	Asian rice	Draft	Midha et al., 2016
	22B	LNPE00000000.1	cheese rind	Draft	None
	CHJ_154	LNPT00000000.1	cow milk	Draft	None
	CCM_2738	MRZO00000000.1	human	Draft	None
	UGA5	NWQI00000000.1	human	Draft	None
	HKUOPL8	NZ_CP007208.1	animal feces	Draft	Ma et al., 2014
	INIFAP_004-15	PIZN00000000.1	Asian tick	Draft	None
	INIFAP_005-08	PIZP00000000.1	Asian tick	Draft	None
	NCTC_11043	PPQM00000000.1	human	Draft	None
	SNUC_3812	PZEK00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_3232	PZEL00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_1397	PZEN00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_966	PZEP00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_233	PZEQ00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_27	PZET00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_5173	QXUC00000000.1	Holstein	Draft	Naushad et al., 2016



	SNUC_4554	QXUF00000000.1	Holstein	Draft	Naushad et al., 2016
	SNUC_237	QXUK00000000.1	Holstein	Draft	Naushad et al., 2016
	SMQ-121	NZ_CP008724.1	NA	Draft	None
	WT6	SPOU00000000.1	mouse	Draft	None
	NCTC_11043	UHEI00000000.1	NA	Draft	None

**Table 4.2.** Wrongly named *Staphylococcus* species were assigned to their right taxonomic position based on pfbA gene and confirmed by WGS comparisons.

Wrong position	Strain	Accession number	Correct position	Host	Genome status	Citation
<i>S. aureus</i>	KUH140087	AP020315.1	<i>S. argenteus</i>	human	finished	None
<i>S. fleurettii</i>	MBTS-1	MWJM01.1	<i>S. vitulinus</i>	cucumber	Draft	Li et al., 2017
<i>S. fleurettii</i>	SNUC_248	PYYE01.1	<i>S. vitulinus</i>	Holstein	Draft	Naushad et al., 2016
<i>S. fleurettii</i>	SNUC_182	QXSE01.1	<i>S. vitulinus</i>	Holstein	Draft	Naushad et al., 2016
<i>S. fleurettii</i>	NCTC13829	UHDL01.1	<i>S. vitulinus</i>	NA	Draft	None
<i>S. hyicus</i>	NCTC8294	CABEIE01.1	<i>S. aureus</i>	NA	finished	None
<i>S. pasteurii</i>	SP1	CP004014.1	<i>S. warneri</i>	NA	finished	None
<i>S. pasteurii</i>	KR	NCXJ01.1	<i>S. warneri</i>	kefir	Draft	None
<i>S. pasteurii</i>	65WT	PUEU01.1	<i>S. warneri</i>	human	Draft	None
<i>S. schleiferi</i>	192	POVG01.1	<i>S. fleurettii</i>	human	Draft	None
<i>S. schleiferi</i>	205	POVI01.1	<i>S. fleurettii</i>	human	Draft	None
<i>S. schleiferi</i>	214	POVJ01.1	<i>S. fleurettii</i>	human	Draft	None
<i>S. schleiferi</i>	ATCC 43808	POVK01.1	<i>S. fleurettii</i>	human	Draft	None
<i>S. schleiferi</i>	NCTC12218	UHEF01.1	<i>S. fleurettii</i>	NA	Draft	None

**Table 4.3.** Species name, length of pfbA gene (bp), pfbA primers designed for the cPCR and/or qPCR to identify to the species level and differentiate 42 *Staphylococcus* species, and the amplicon sizes (bp).

No	Species	pfbA gene size (bp)	Designed primers (5' to 3')		Amplicon size (bp)
			Forward	Reverse	
1.	<i>S. agnetis</i>	1110	GCGTAATACGCCAGCACAGTTG	CACTTATGGCATTCAACGWGCA	748
			GCATGTGAGCCAATTGCGC	GGCGGTACTTTTGATATGAATGG	368
2.	<i>S. argenteus</i>	1098	CCGTCCCTYACTCCTAGATATC	ATCGCAAGCAGACAACRGTG	730
			GCATGTGAGCCAATAGCCC	CGACGTTGTAATTCTGGAACGC	458
3.	<i>S. arlettae</i>	1089	CGAATCCACCTGCACACTG	GTGGGACGCAATAAACGTAAAG	770
			GCATGCGAACCAATAGCACG	CGTGGTGGTGTCTAGAYATG	371
4.	<i>S. aureus</i>	1086	CTTGAATACCCGCTTCAAYAGGA	CRTGCGTTAAATCRTGGAAGATG	994
			CTTGCATGTGAGCCAATAGC	CGYCGATGTCATTCKGGTCC	458
5.	<i>S. auricularis</i>	1117	TGTCATACCCCTGTTGGTTCG	CTGAGAAAGGGAAGAGATGC	656
			GATCATAGCGACTGGCATG	GGCTATGACGGTAACAGTCAC	416
6.	<i>S. capitis</i>	1086	CSCCAAGAAATCGTATACCACC	GCGAGTAGGGAAAGATGCATTG	611
			GCRCGATTCCAYGCTTTC	GGTCATGCTGAAGATATTCARC	293
7.	<i>S. caprae</i>	1086	CGTTAAAGTTCTCACCTGCTTGC	AGCACGGGGAACATACCG	795
			GCGTGTGAACCAATCGCTC	CTTCGATATGAATGGGGGAGA	360
8.	<i>S. carnosus</i>	1113	CGCCTTGAGGGCCAGTAAC	CACGAAAGCATTGCAACGCGC	818
			GCATGTGACCCGACAGCAC	TCGGCGGGACATTTGATATG	370
9.	<i>S. chromogenes</i>	1104	CACTTTGTTGACGTAKTTCCGG	GATACGATTGGGATTCAACGYGC	1027
			GCACCTGCACAATTCTTGAASG	GGGGGCACCTTCGATATGAA	506
10.	<i>S. cohnii</i>	1083	GCACTCGAATKGCATCTTTAGAC	GGGCAGATACACGYGCGATTC	888
			GTTATCGTACCATCCGTKGCACC	GGAGAGTATCATATWCGTAGAYCG	446
11.	<i>S. condimenti</i>	1113	GTACCAAGAAAGCGAATACCTC	CGGATGGCGTTATGGACAATACC	582
			GCATGTGACCCGACAGCAC	CGGCGGCACTTTTGATATG	369

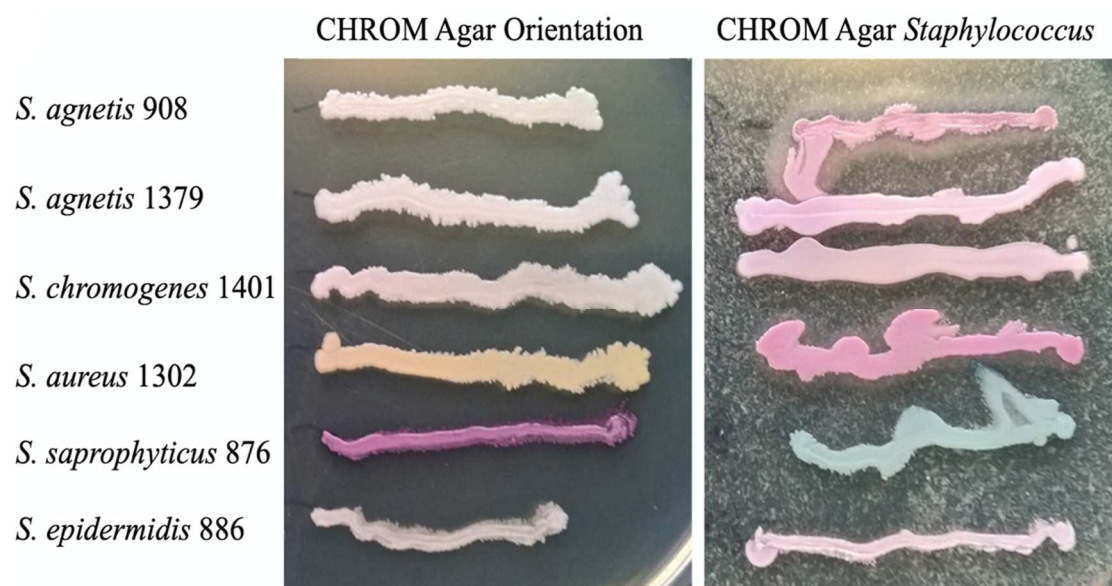
12	<i>S. delphini</i>	1104	TGTAAGGGTAAWGGACGCCA	GGGGCATACAACGTGCGCTCA	267
			CAACAATGCATCTTTGCCAGTCC	CCTAGCGGTACTTATCATATYGC	108
13	<i>S. devriesei</i>	1083	GGCCAGTTAATGGGTCAGMCGC	CCAGTTAATCCGTAATCTTGTGCG	836
			GTGACGACTTGCATGKGAACC	CCCATTTCATATCAAATGTTCCGCC	378
14	<i>S. epidermidis</i>	1098	CGTAATCGTTCCATCTGTAGTTC	GGCAGACACACTGTTTATATAACC	474
			C	ACAGCCATGTGCATGGGACA	197
15	<i>S. equorum</i>	1059	GCTTGAGTTGATCTTTGTTTCCC	CCTAAATTTGGGACGACAGACGG	329
			ACACCRCCAGCACAGTTT	GGCAATTCAGATACGCCTACG	197
16	<i>S. felis</i>	1101	GCAATTTGTTGGTCTTTGACGTG	GTAAAGACGTGATTTGGSATACG	1061
			GCATGYGACCCTATCGCAC	GGGTGGCACATTTGATATGAA	369
17	<i>S. fleurettii</i>	1107	GAGTTGACTGACATCGTGTTG	CGTTGAATGCCGATCGTGTCGCGC	1039
			CATTCCCGATGATATAGACACC	CCACAAGCGTCCACACCATGCCC	580
18	<i>S. gallinarum</i>	1085	CGACTGGCATGCGARCCGATAG	GGATATCTTGCGCATGTCCAATAC	320
			C	GCCACATGCATCTATTGCATGACC	222
19	<i>S. haemolyticus</i>	1083	CRTCTTCACCCTGGTTTAGGG	CACWGTACTTATACCTAGCGGTAC	953
			G	CTGCGATGTCAATTGGACATGC	322
20	<i>S. hominis</i>	1080	G	GATCAATGCACAGGATTTTGG	402
			GCATGTCCACCTACAACATCAAG	CCGCAATGTCTATAGGYCATGC	322
21	<i>S. hyicus</i>	1101	TATTAACGTAGCGATGTGGTG	TACGATTGGAATACAACGGGCA	905
			GCATGCGACCCAATCGCTC	GGCGGTACGTTTGATATGAA	368
22	<i>S. kloosii</i>	1083	GCACGCTCATCTATATCTTGC	GACGCGCGCAATTTGGA	1049

			GCATGTGAACCAATCGCCC	CGCCAAGGCAAAGATGCG	455
23	<i>S. lentus</i>	1128	GTGGAACCAGACTGTGCACC	GAAAATCTCCTAGCGCTATGTTG	673
			GCATGTGACCCAATAGCTC	GGTGGCACTTTTGATGCATA	368
24	<i>S. lugdunensis</i>	1086	CCAAGGACCCATGCCAGCTG	GCCACTGTATTGATGCTTGTGG	230
			GCATGAGARCCAATCGCCC	GAGATGTAGATGGAGATCGCTGG	187
25	<i>S. lutrae</i>	1107	CGTCCCGTCATTGACACC	GTTGTTGGAGGCCATGGC	171
			CGCGCCTTCAACGAACAA	GCTTGTGGACTGGATGGTG	117
26	<i>S. muscae</i>	1098	ACCTCCCACACATCGGTG	GGCTCACACGCCAGTCGTG	150
			CCCACACATCGGTGAAAGAC	CGCCAGTCGTGTTGATCA	138
27	<i>S. nepalensis</i>	1089	AGGTGTGAGCGCGTATTGGTTC	CCTAAGTTTGGCGCTACTGATGGG	201
			AGCGCGTATTGGTTCATGCC	CGCCTCATATGGATGCATGG	118
28	<i>S. pasteurii</i>	1095	GTCTTCAAGATGTATCTTCTGTG	ATGYGAAAGACTTTGGCGCAGAAG	1010
			CAGCAGCATTCTTGCCATCC	CTGCGGTGTGTGTTGGTCATGC	501
29	<i>S. pettenkoferi</i>	1089	GACTGCGCTTCACGTACTTGGC	GAAGGTACGACACTWCAAATGGA TG	710
			GCGTGAGAACCGATAGCAC	ACGCGCATGCCCWACACAGAG	124
30	<i>S. piscifermentans</i>	1113	AGTTTGACTTGCTTGGCTGATAG T	TGATTATCAACCCCGTTCAATTCTG	1075
			GCGTGAGAGCCGATAGCGC	GAACGGTTCACGCATTCCAG	351
31	<i>S. pseudintermedius</i>	1107	TGTCATGTACACCTGTTAATTGC G	TGGGGCATAACAGCGTGCGCTC	970
			AACGATCGAAGCGTGTCGC	GTAGGRGGTCATGGGATAGATGC	276
32	<i>S. saprophyticus</i>	1083	CCTGCTTGTGTCCCCCTTTC	GGGGAACACAAATGGGCAG	836
			CGGTTCCAAGGTTGCATACCGGG	GGTGGCACGTTTGATATGAATGGG	350

33 .	<i>S. schleiferi</i>	1107	GGTGTGAGCGCATAATCTTGCG	CAATATGAATGGTGTGCACTATCC	438
			CGKCTTGCAATGTGAACCAATTGC	TGGGCATGGTGTGGACGCTTG	261
34 .	<i>S. schweitzeri</i>	1098	GCCAGGTACTTGAATATCTAGCT G	TCATGCTCTTGATGCGTGTGG	127
			CTAGCTGTATYGCTTCAGAGT	GATGBGTGTGGTATTAATGGAC	100
35 .	<i>S. sciuri</i>	1107	GACCCTGCTTGTCCGCCTCTAT	GCRATTGATGCATGTGGCTTAG	464
			GTCCGCCTCTATTTAATTTATGG	GGAAATTCAGGTGATCCRAACAT	262
36 .	<i>S. simiae</i>	1086	CGGTGTTAGCGTATAATCTTGC	ACAAAGGATACAATGGTCATGGCC	488
			CTCGCATGAGAACCAATCGCTC	GGTGGTGTCTTTGATATGAATGGC	371
37 .	<i>S. simulans</i>	1119	GTGTGCTTTAAAAGGTGTCAACG C	GGGATGCGAAATGGACTTTATC	519
			TGCATGTGAACCGATTGCGCG	ATCGGCGGTACATTCGATATG	372
38 .	<i>S. stepanovicii</i>	1107	GTGTAACACGACATGCATTTGC	GCAATGCTTAAAAATGGCCACAGC	550
			GCGTGTGAACCAATGCCCC	GGTGGAACATTCGATGCATATGG	368
39 .	<i>S. succinus</i>	1083	GTGTGTTTCGACTTCAGAGGTG	GCAGCGCGCATTAAATTATGC	690
			CGGCTAGCRTGTGAACCAATTGC	GATGAGTATAGGTCATGCCCA	324
40 .	<i>S. vitulinus</i>	1104	GTTGACCCTGCTTGGCCACCTC	CCATGACCATCGTACCCATAGT	625
			CCATGCTGACATGTTTGGATCTC C	GCGTGTTCCTACTGTCGTCACGG	166
41 .	<i>S. warneri</i>	1095	CCAGTATAAGGATCYGCTGCG	GCAAAGATGCACTRTTAAAGAATG	640
			CGTATGCCCCCTACACAATT	GGGRCATGCAGAAGACATTCAA	453
42 .	<i>S. xylosus</i>	1089	TGTGAGCCWATTGCACGATTCC	GGGACACGCAGAAGATATTTCAG	306
			GAGCCWATTGCACGATTCCA	GACACGCAGAAGATATTTCARATAC	301

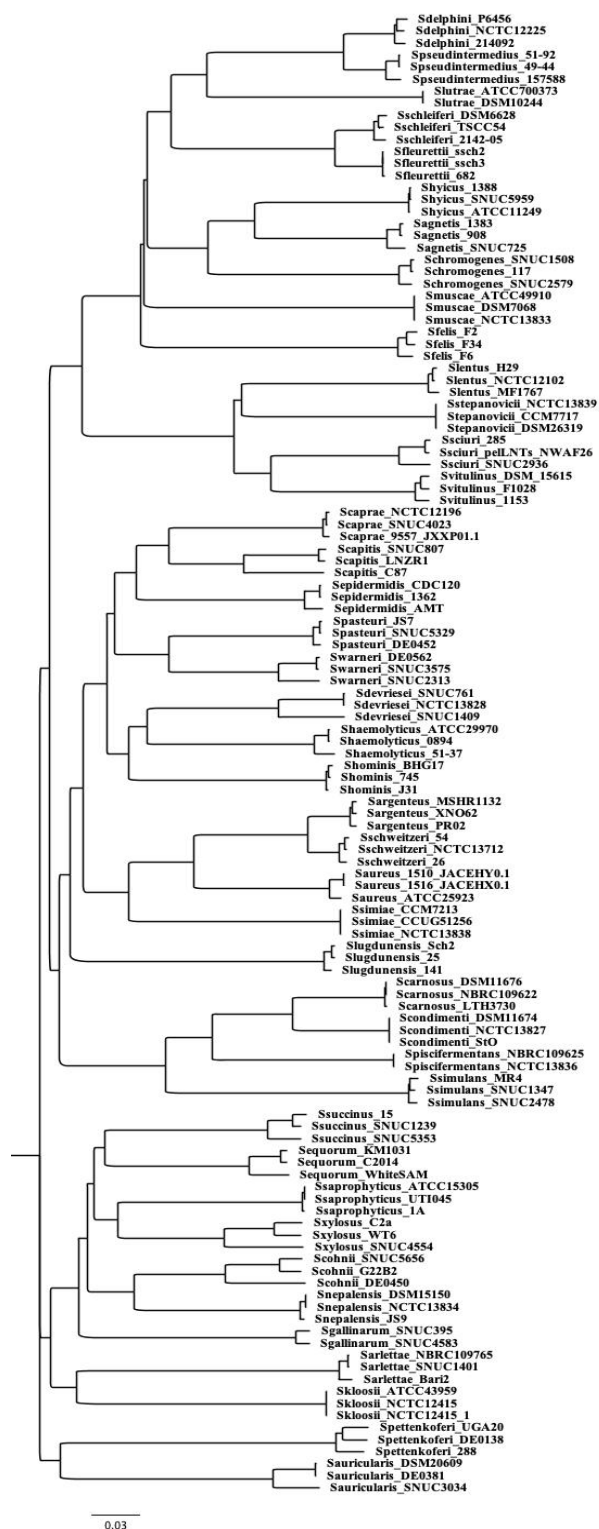
**Table 4.4.** Nine *Staphylococcus* species specific pfbA (F & R) pfbA primers utilized empirically for the cPCR and qPCR to identify to the species level and differentiate 9 *Staphylococcus* species, and the amplicon sizes (bp).

No	Species	Suggested primers (5' to 3')		Primers name	Amplicon size (bp)
		Forward	Reverse		
1.	<i>S. chromogenes</i>	CACTTTGTTGACGTAKTCCGG	GATACGATTGGGATTCAACGYGC	chr_pfbA-FxR	1027
2.	<i>S. aureus</i>	CTTGAATACCCGCTTCAAYAGGA	CRTGCGTTAAATCRTGGAAGATG	aur_pfbA-FxR	994
3.	<i>S. hyicus</i>	TATTAACGTAGCGATGTGGTGC	TACGATTGGAATACAACGGGCA	hyi_pfbA-FxR	905
4.	<i>S. cohnii</i>	GCACTCGAATKGCATCTTTAGAC	GGGCAGATACACGYGCGATTC	coh_pfbA-FxR	888
5.	<i>S. saprophyticus</i>	CCTGCTTGTGTCCCCCTTC	GGGGAACACAAATGGGCAG	sap_pfbA-FxR	836
6.	<i>S. agnetis</i>	GCGTAATACGCCAGCACAGTTG	CACTTATGGCATTCAACGWGCA	agn_pfbA-FxR	748
7.	<i>S. lentus</i>	GTGGAACCAGACTGTGCACC	GAAAATCTCCTAGCGCTATGTTG	len_pfbA-FxR	673
8.	<i>S. capitis</i>	CSCCAAGAAATCGTATACCACC	GCGAGTAGGGAAAGATGCATTG	cap_pfbA-FxR	611
9.	<i>S. epidermidis</i>	CGTAATCGTTCCATCTGTAGTTCC	GGCAGACACACTGTTTATATACC	epi_pfbA-FxR	474

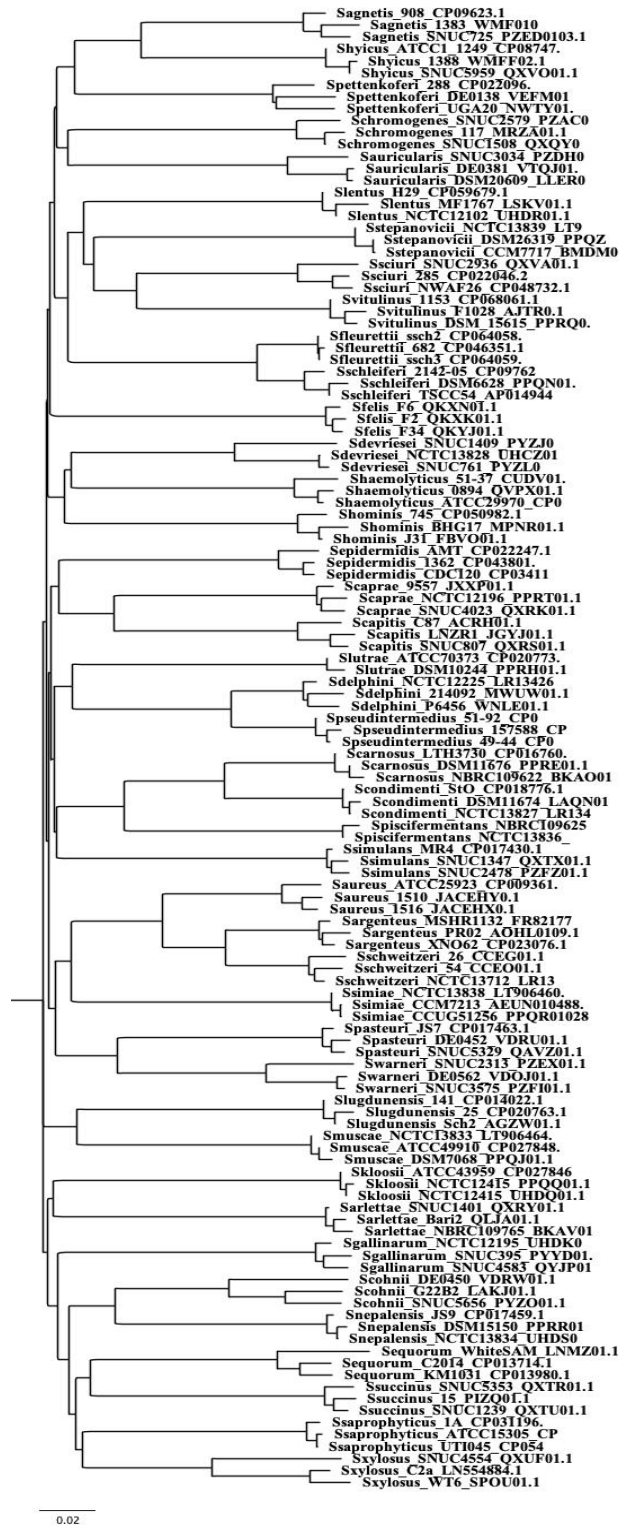


**Figure 4.1.** Diagnosis of a number of *Staphylococcus* based on Chrome Agar media.

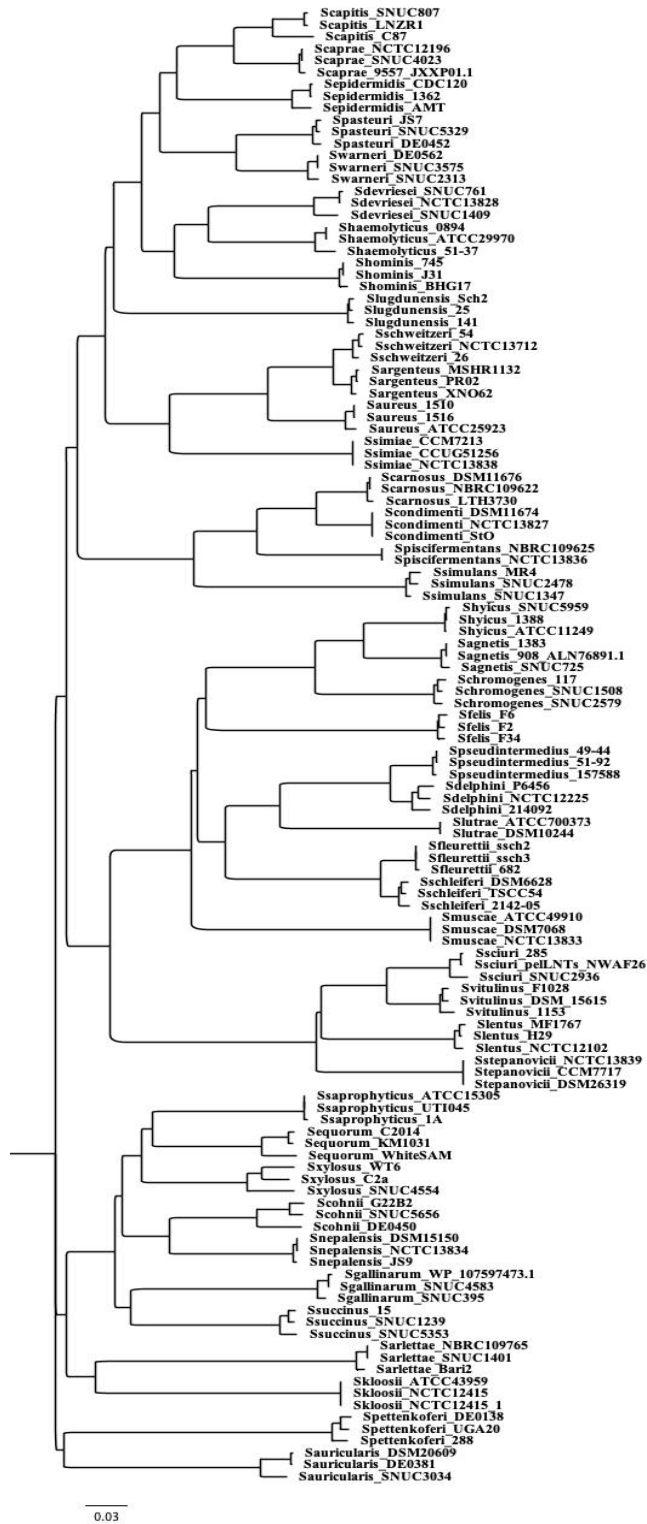




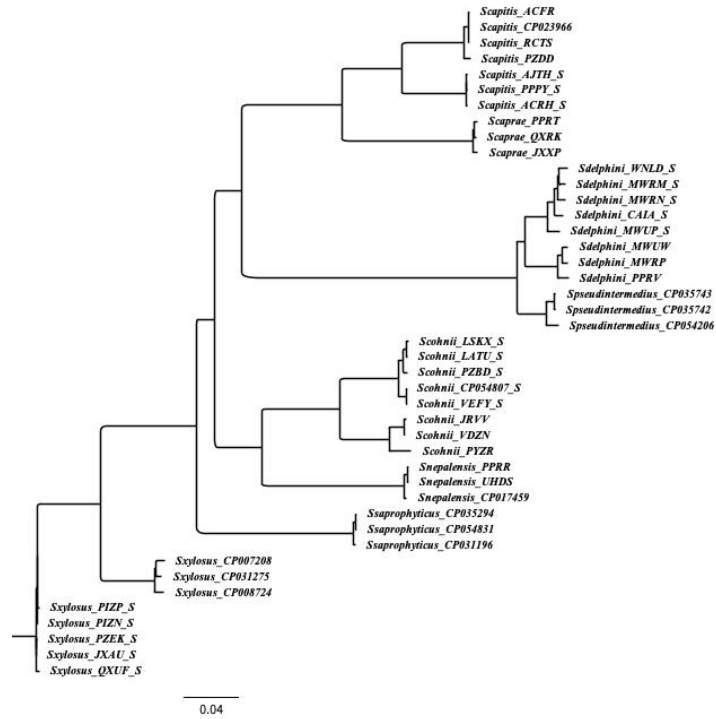
**Figure 4.2.** Neighbor joining tree using pfbA NTs for 42 species of *Staphylococcus* each with 3 strains.



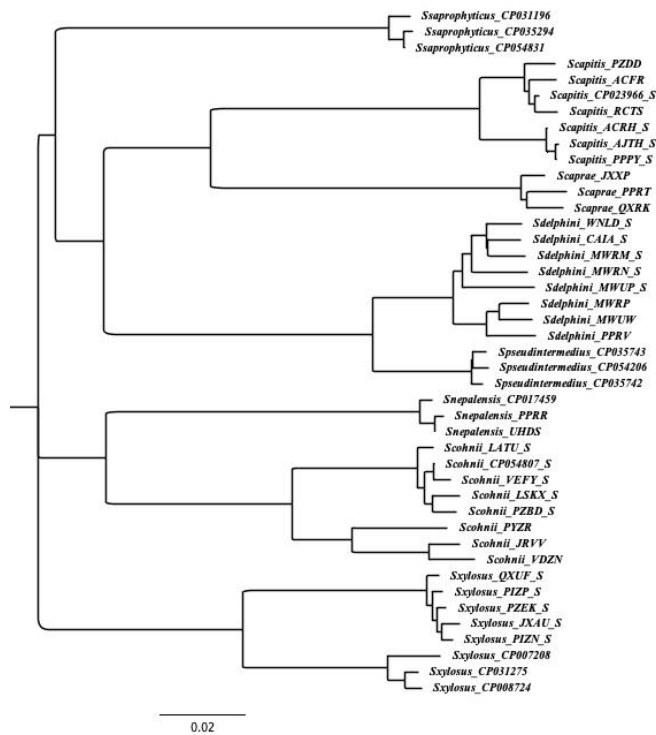
**Figure 4.3.** Neighbor joining tree using WGS for 42 species of *Staphylococcus* each with 3 strains.



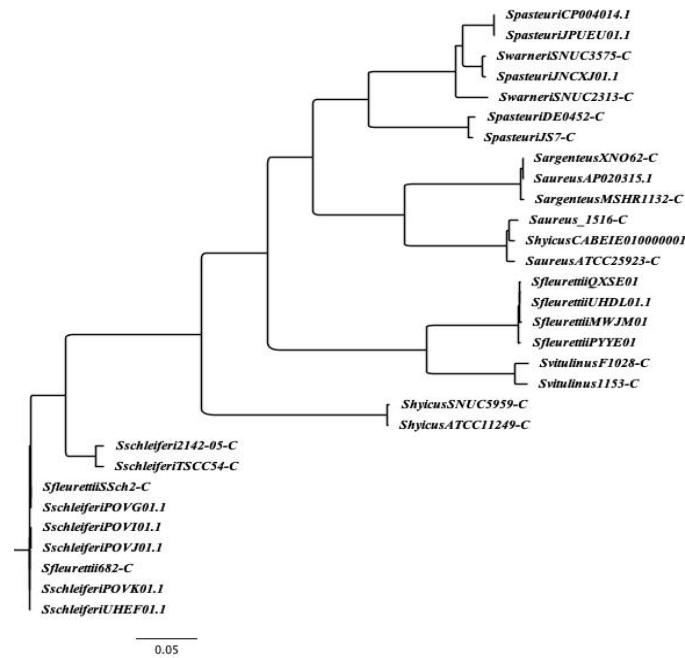
**Figure 4.4.** Neighbor joining tree using *pfbA* proteins for 42 species of *Staphylococcus* each with 3 strains.



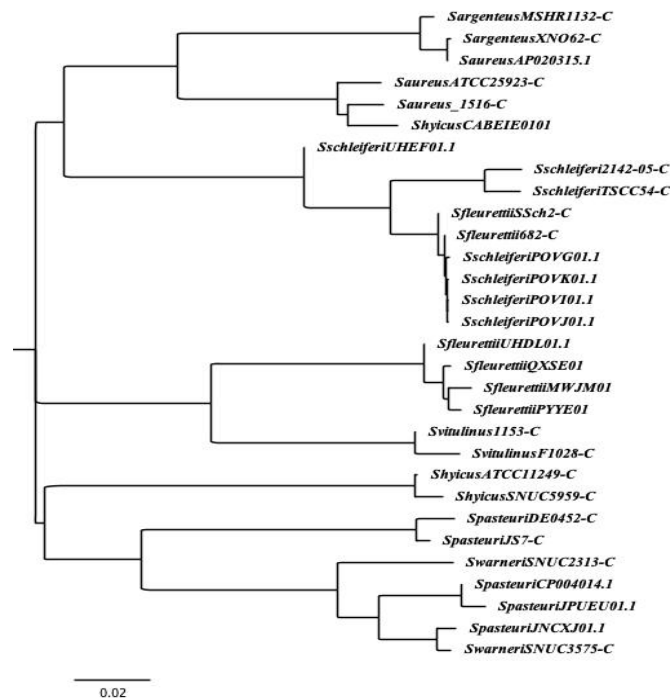
**Figure 4.5.** Neighbor joining tree showing subspecies of 5 *Staphylococcus* species based on pfbA gene. S shows the position of the sub-species.



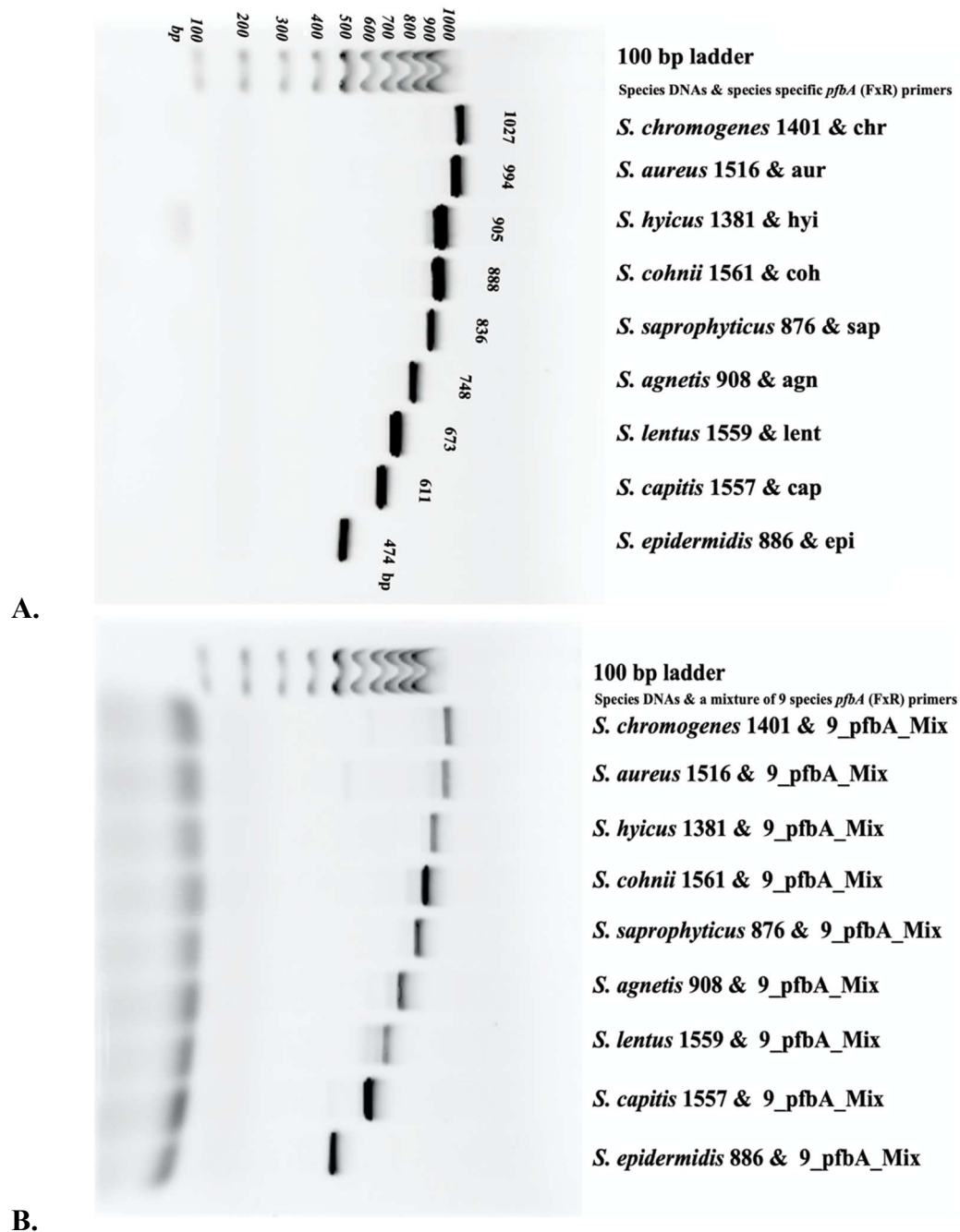
**Figure 4.6.** Neighbor joining tree showing subspecies of 5 *Staphylococcus* species based on WGSs. S shows the position of the sub-species.



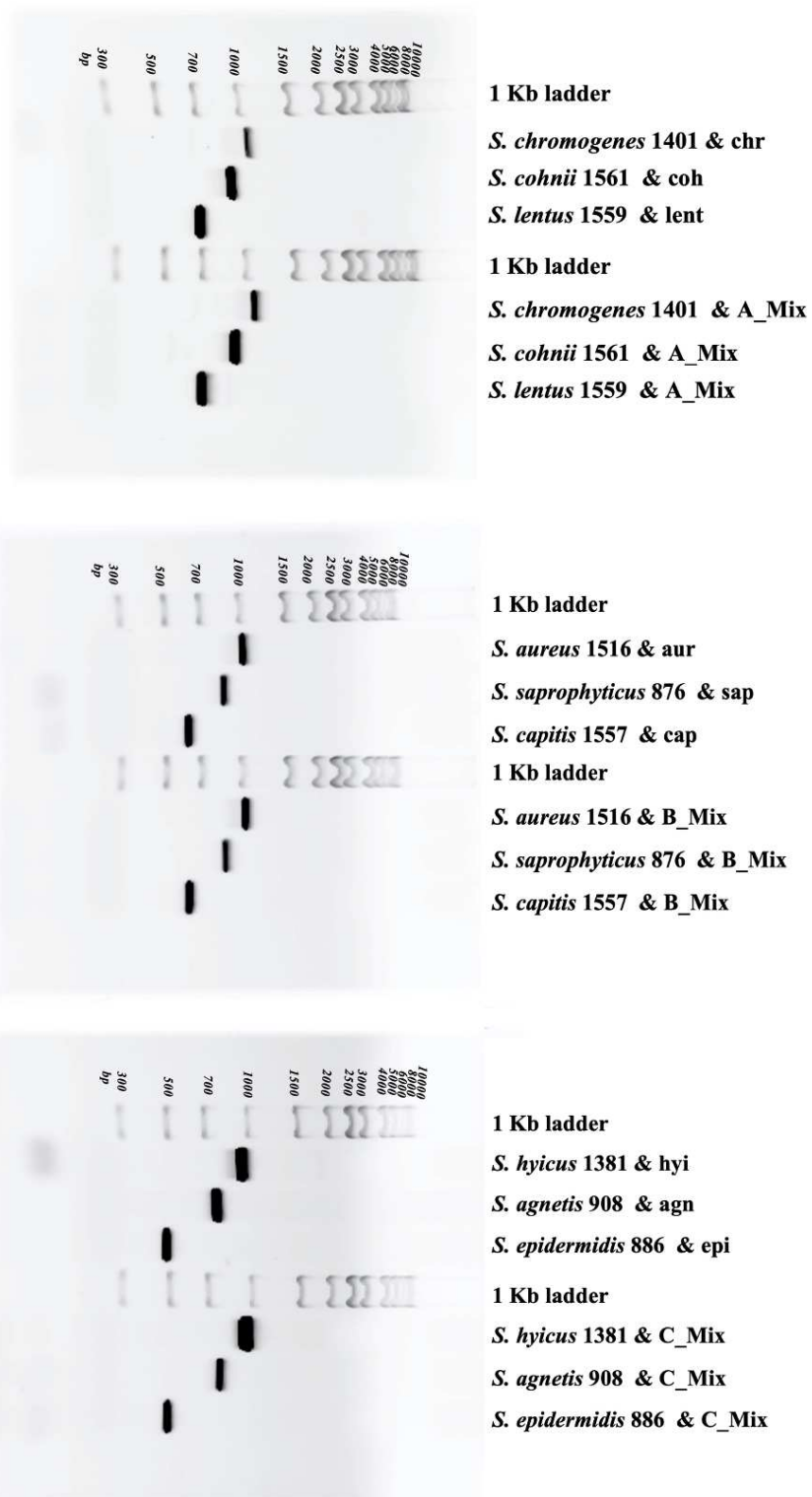
**Figure 4.7.** Neighbor joining tree using 14 wrongly named *Staphylococcus* species that have been re-assigned to their right position using pfbA gene. -C is used for correctly named.



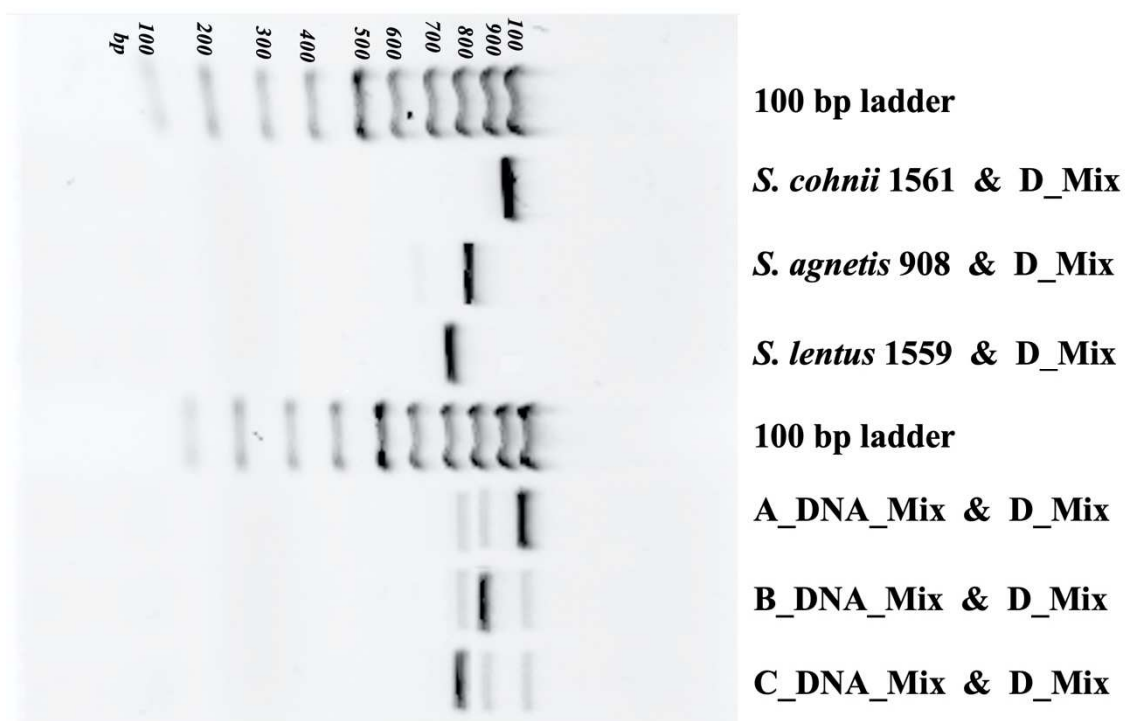
**Figure 4.8.** Neighbor joining tree using 14 wrongly named *Staphylococcus* species that have been re-assigned to their right position using WGS. -C is used for correctly named.



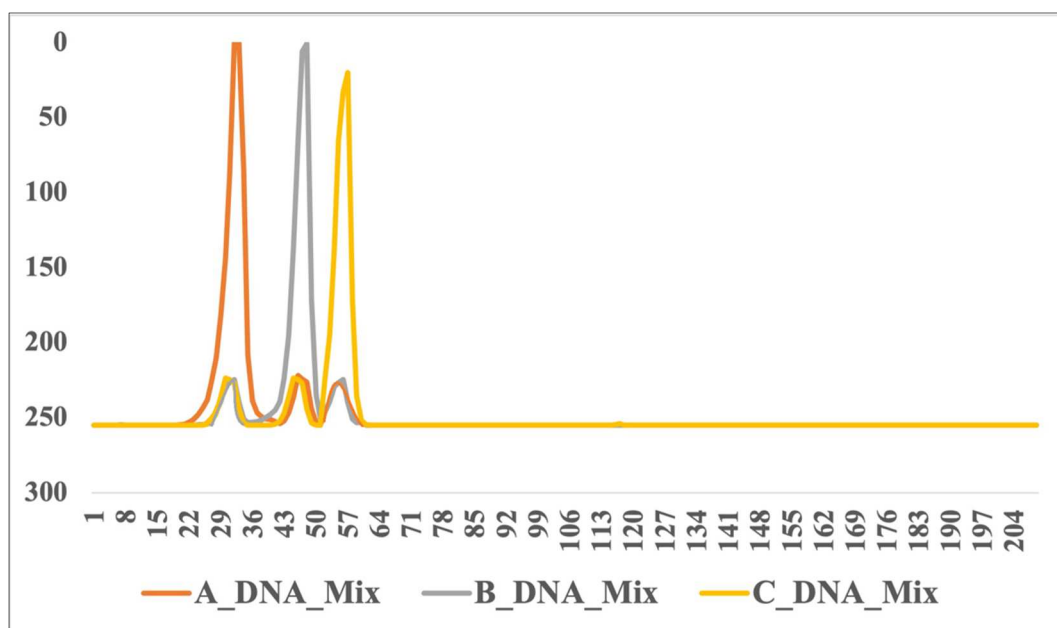
**Figure 4.9.** Gel images showing 9 *Staphylococcus* species DNAs amplified using individual species specific (A) and a mixture of 9 species *pfbA* (F & R) primers (B).



**Figure 4.10.** Gel images showing *Staphylococcus* species DNAs amplified using individual species specific and 3 mixtures (A,B,C) of 9 species pfbA (F & R) primers.

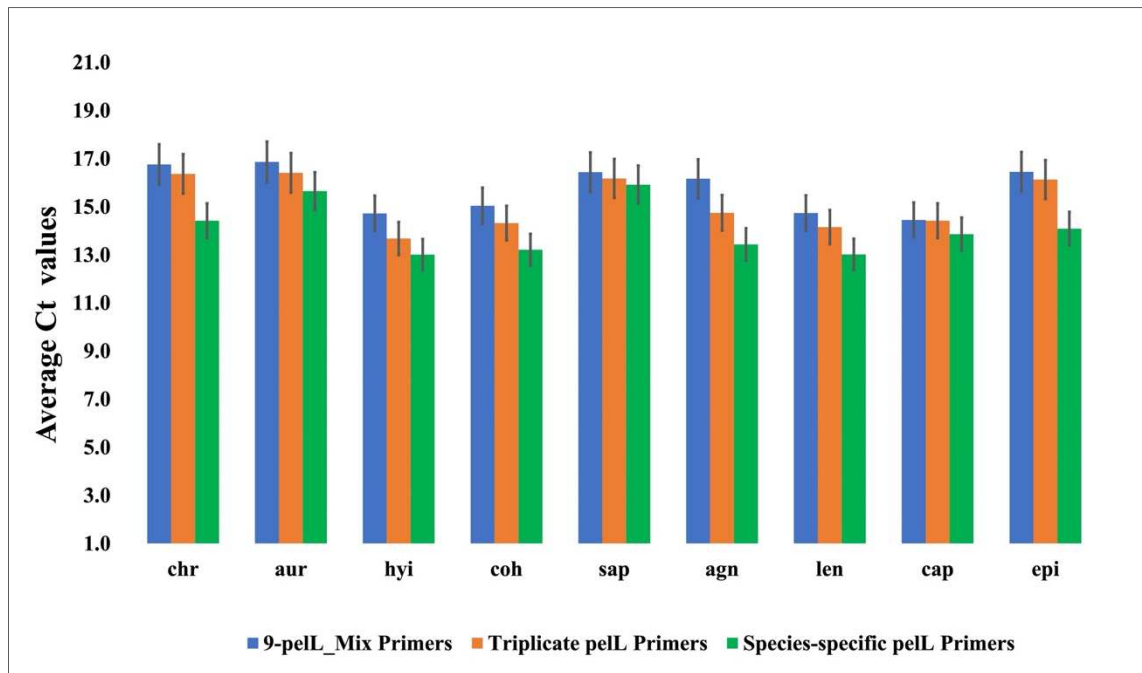


**Figure 4.11.** Gel image showing 3 *Staphylococcus* species different DNAs concentrations (*S. cohnii*, *S. agnetis* and *S. lentus*, respectively) amplified using a mixture (D\_Mix) of 3 species pfbA (F & R) primers.

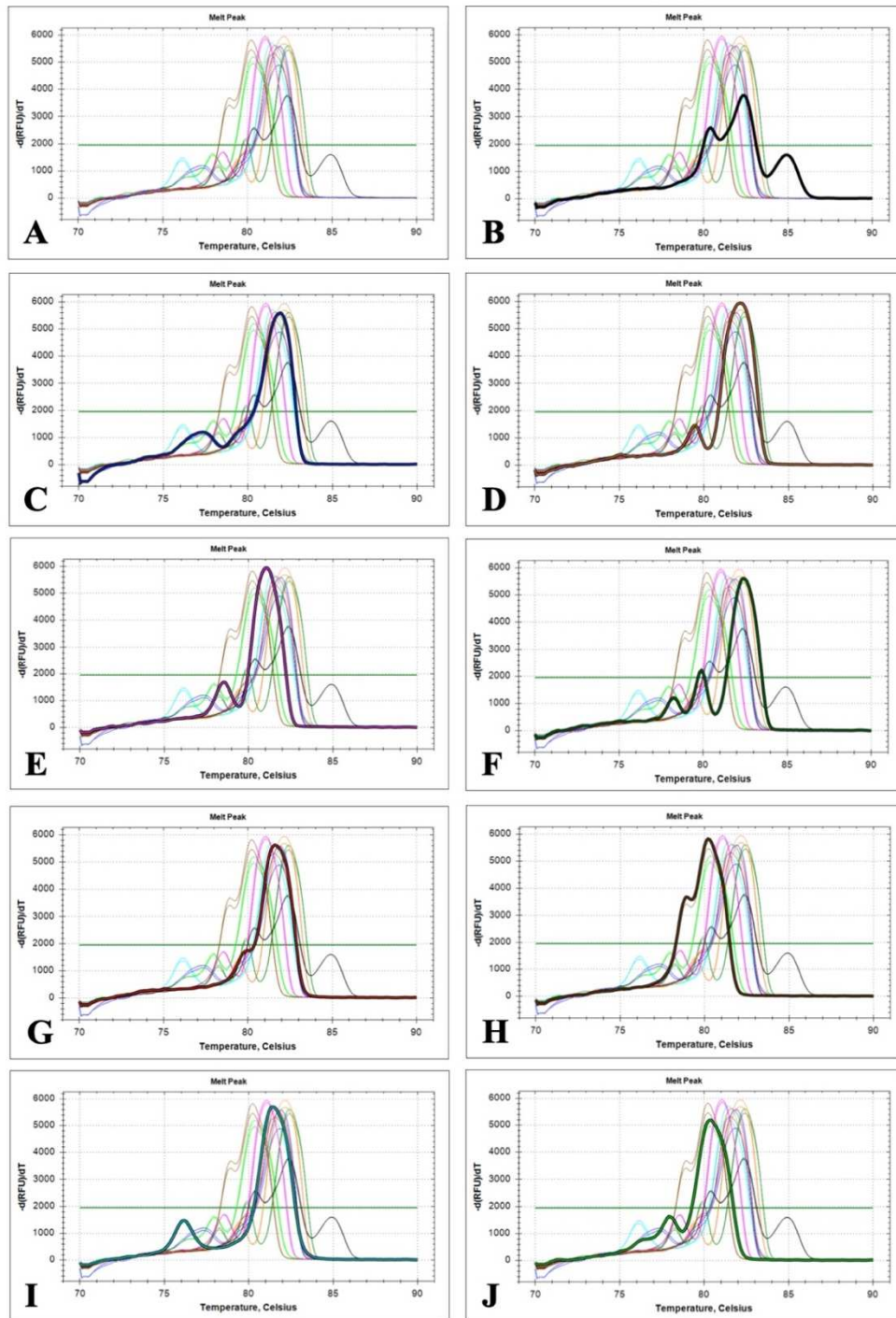


**Figure 4.12.** Densitometry plot shows that a combination (D Mix) of three *Staphylococcus* species pfbA (F and R) primers was used to amplify three different DNA concentrations (*S. cohnii*, *S. agnetis*, and *S. lentus*).

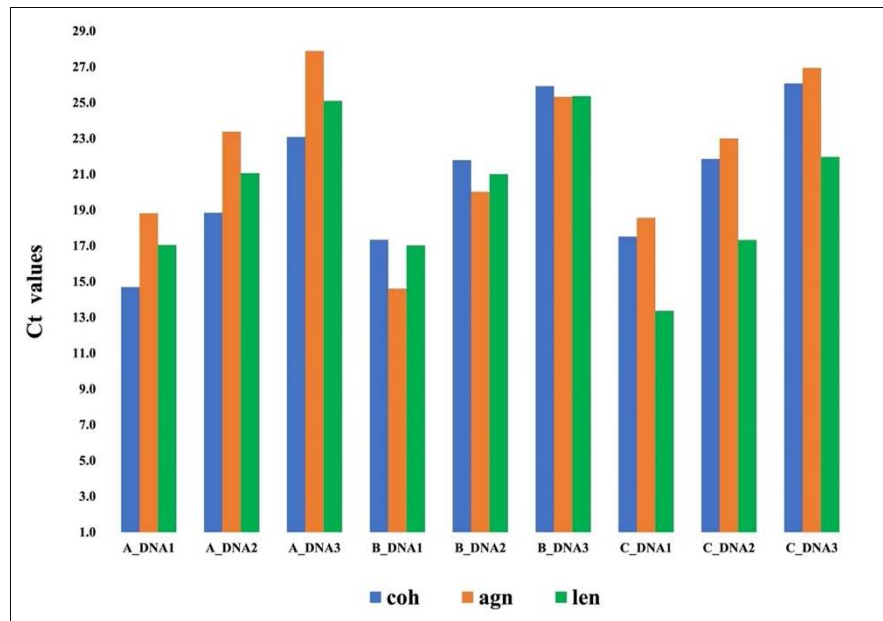




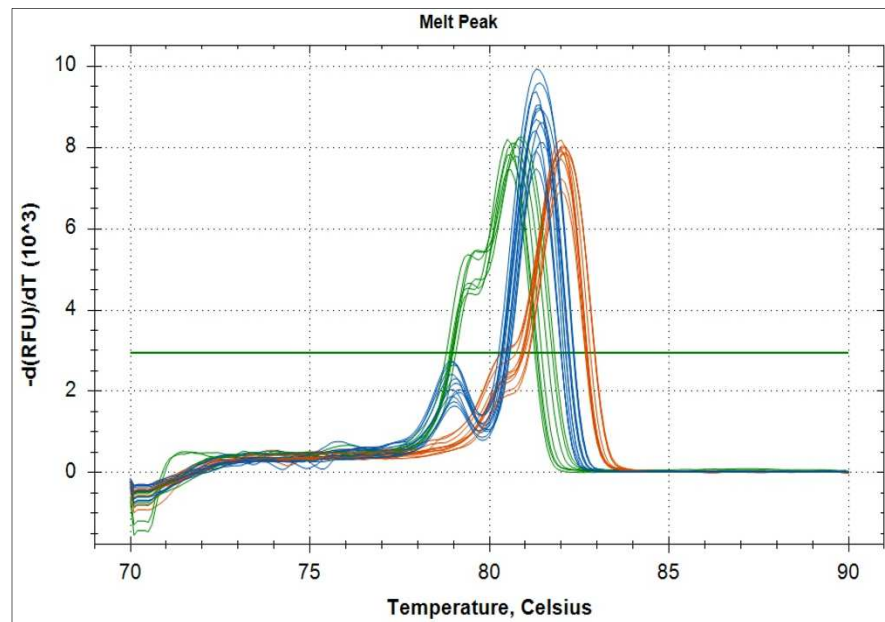
**Figure 4.13.** qPCR results using pfbA (F<sub>x</sub>R) primers of 9 *Staphylococcus* species; all 9 pfbA primers in one mixture; in triplicates every 3 pfbA primers in one mixture, and species specific pfbA primers used to amplify individual DNA (0.25 ng/ul) of the 9 species.



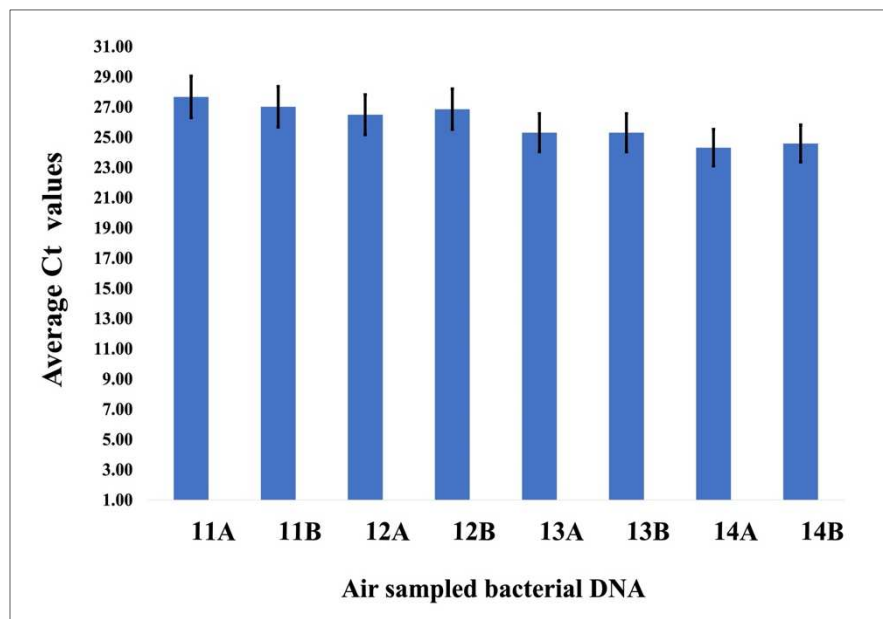
**Figure 4.14.** Comparison of HRM curves of the extracted DNA of 9 *Staphylococcus* species, in triplicates, amplifying the *pfbA* gene of every species using *Staphylococcus* species specific *pfbA* (FxR) primers; A) All HRMs together. B-J) Highlighted HRM peaks of *S. chromogenes* (chr), *S. aureus* (aur), *S. hyicus* (hyi), *S. cohnii* (coh), *S. saprophyticus* (sap), *S. agnetis* (agn), *S. lentus* (len), *S. capitis* (cap), and *S. epidermidis* (epi), respectively.



**Figure 4.15.** qPCR results using coh, agn, and len pfbA (FxR) primers for 3 DNA mixtures (A, B, and C) consisted of different amounts (80:10:10; & 10:80:10; & 10:10:80) of 3 *Staphylococcus* species, *S. cohnii*, *S. agnetis*, and *S. lentus*. Each DNA mixture (0.25 ng/ul) has been diluted in 3 successive 10-fold dilutions (1x, 0.1x, and 0.01x).



**Figure 4.16.** Comparison of HRM curves of 3 *Staphylococcus* species DNA out of 9 *Staphylococcus* species amplified using their species-specific pfbA (FxR) primers; *S. lentus* (green), *S. cohnii* (blue), and *S. agnetis* (orange).



**Figure 4.17.** qPCR results using *S. lentus* pfbA (FxR) primers for air sampled bacterial DNA amplification.

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## **CHAPTER 5**

**A simple rapid inexpensive method for DNA extraction from environmental samples.**

## **Chapter 5: A simple rapid inexpensive method for DNA extraction from environmental samples.**

### **Abstract.**

DNA extraction for downstream molecular diagnostic applications can be an expensive, time-consuming process. We devised a method to quickly extract genomic DNA from environmental samples based on sodium hydroxide lysis of cells with or without capture by magnetic beads. We investigated the impact of different NaOH concentrations on subsequent quantitative PCR. NaOH extraction was effective for Gram-negative and Gram-positive bacteria. We applied the optimum NaOH concentration to different environmental, agricultural, food and clinical samples, including air, soil, sewage, food, laboratory surfaces, and chicken cloacal swabs. Bacterial DNA could be assessed by qPCR by dilution of the bacterial NaOH lysate or the DNA captured from the NaOH solution by magnetic beads. Two kinds of paramagnetic beads were tested and we describe an effective bead binding buffer. The final DNA extraction method using NaOH is extremely low-cost and can be completed in 10 minutes at RT. This technique is well-suited for high throughput analyses and allows for the extraction of bacterial DNA in resource-limited situations.

## **Introduction.**

Reliable and accurate molecular diagnostics are crucial for the detection of pathogenic bacteria in clinical, food, and environmental samples (Kralik & Ricchi 2017; Deshmukh et al., 2016; Deurenberg et al., 2017). In the study of bacterial genomes, one of the most important steps is the isolation of high-quality genomic DNA. There are various methods and commercial kits available for the rapid extraction of bacterial DNA from bacterial cells for polymerase chain reaction (PCR) (Sambrook et al., 1989; Maloy, 1990; Nelson & Krawetz 1992; Ausubel et al., 1994). To identify genomic sequences, the cells need to be lysed by heat treatment, physical, detergent, enzymes, pulverization, pH, chaotropic chemicals, or a combination of these, which is then followed by either chaotropic-based fractionation, phenol extraction, or size exclusion column purification (Kolm et al., 2017; Chapela et al., 2015; Law et al., 2014; Barbosa et al., 2016). Purification of the extracted DNA can involve precipitation or column-based nucleic acid capture. Traditionally, DNA extraction from cultured bacteria often included expensive, and complicated enzyme combinations or costly hazardous chemicals such as phenol, or chloroform (Martzy et al., 2019). These methods vary in effectiveness depending on the application and the matrix from which the cells are obtained (Reischer et al., 2008). Although current techniques are well proven to produce high-quality nucleic acids (DNA or RNA), they are time-consuming, labor-intensive, and expensive to implement. In addition, the quantity of genomic DNA that they produce could be insufficient (Tan & Yiap, 2009). These limitations become much more significant in settings with limited resources. As a result, the implementation of molecular testing is considerably hampered in many regions of the globe, especially in developing countries (Martzy et al., 2019). Therefore, the development of rapid, easy-to-use, and more efficient DNA

extraction techniques that do not rely on complex laboratory equipment facilitates the advancement of molecular clinical detection technologies.

Recent years have seen the development of novel approaches for extracting DNA from plants (Wang et al., 1993), mouse tails (Truett et al., 2000), microbes from sediments (Kouduka et al., 2011; Morono et al., 2014), and fungal and oomycetes samples (Osmundson et al., 2012) using NaOH alone or in combination with other chemicals. Therefore, it has been proven that NaOH is capable of effectively lysing a variety of cell types and separating them from diverse biological materials within 10 minutes. The extraction would be most effective using NaOH, which would also inactivate nucleases during the extraction process (Wang et al., 1993; Osmundson et al., 2012). In most of these treatments, NaOH was administered along with other chemicals, such as sodium dodecyl sulfate or sodium acetate (Park et al., 2016; Wang et al., 2020). NaOH was used at high temperatures up to 70°–98°C to disrupt cell walls, especially in the case of the cell wall of Gram-positive bacteria (Morono et al., 2014; Vingataramin & Frost 2018). Hence, these NaOH treatments increase the presence of inhibitors in subsequent PCR and make extraction protocols time-consuming and tedious. Consequently, a fast DNA extraction procedure that consistently produces high DNA yields and can be applied to both Gram-positive and Gram-negative bacteria without the use of additional chemicals or enzymatic treatments would be beneficial in terms of saving time and money, while allowing for increased DNA yields.

The goal of this research was to develop an inexpensive NaOH-based technique for rapid lysis of bacteria that can be carried out with minimum laboratory equipment and used for subsequent DNA-based diagnostics. In this research, we used *Enterococcus cecorum* as a model organism for Gram-positive bacteria to investigate the cell lysis because it was the most

recalcitrant bacteria we have ever used for rapid extractions by boiling. The bacterial lysate was liberated with dilute NaOH followed by direct purification using paramagnetic beads. We examined two brands of commercial paramagnetic beads, in different buffer solutions. Also, we have used a cheap and effective protocol for making bead binding buffer suitable for the beads (BioChain Institute Inc., 2022). This study shows a technique that starts with NaOH lyse and is followed by paramagnetic beads or a simple 1:5 dilution in Te. The method was used on different environmental samples, including air, soil, sewage water, food, environmental surfaces, and chicken cloacal swabs. The newly optimized DNA preparation technique could be a substitute for the current expensive protocols, and commercial DNA extraction kits for the detection of various bacterial targets after adjusting the reaction parameters in terms of NaOH concentration, buffer system, incubation time, and temperature.

## **Materials and Methods.**

### **Bacterial media.**

CHROMagar Orientation (CO) and CHROMagar *Staphylococcus* (CS) (DRG International, Inc., Springfield, NJ) as well as Tryptic Soy Broth were used as agar medium (Difco Laboratories, Franklin Lakes, NJ). According to the manufacturer's requirements, the medium was prepared.

### **Bacterial cultures.**

Bacterial strains are listed in Table 5.1. Bacterial stocks were stored at -80 °C in 40% glycerol. Media included: Tryptic Soy Broth (TSB) or Agar (TSA), ChromAgar Orientation (CO), Nutrient Agar (NA), and ChromAgar *Staphylococcus* (CS).



### **Cell density estimation by densimetry.**

Overnight cultures of *Staphylococcus agnetis* 908 were diluted 1:100 in broth. Absorbance at 650 nm was used to compute the cell density based on a pre-calibrated CFU/ml formula  $A_{650} \times 10^9 + 10^6$  (Fig. 5.1).

### **Environmental bacterial specimens.**

#### **Air samples.**

Air samples were collected from one of the University of Arkansas poultry research facilities. The air was collected through 20 ml of 0.9% saline in 30-ml sterile glass impinger (Chemglass Life Sciences LLC, Vineland, NJ) using a portable pipet pump for 20 minutes. The airborne microorganisms collected in the 0.9% saline were then centrifuged at 5000 x g for 10 minutes at 4 °C. The pellets were re-suspended in 100 ul of autoclaved sterile pure water (SPW).

#### **Soil samples.**

Soil was collected on the University of Arkansas campus. The top dried layer of the ground was removed to a depth of 2–5 centimeters using a sterile spatula. The wet, silty soil was collected in a sterile 50-ml conical tube. Fifty mg of each soil sample was transferred into a 1.5 ml tube. The soil was resuspended in 450 ul of SPW. The soil suspension was vortexed for 30 seconds.

**Chicken cloacal specimens.**

Cloacal bacteria were swabbed from day old chickens using sterile cotton swabs at the University of Arkansas poultry research facilities. Each swab sample was swirled in 450 ul of SPW in a 1.5 ml tube.

**Food samples.**

Cheese surface was swabbed using sterile cotton swabs then subsequently swirled in 450 ul of SPW in a 1.5 ml tube. Bread samples were 10 mg in 450 ul of SPW in 1.5 ml tubes. In both cases the tubes were vortexed for 30 seconds.

**Environmental surface samples.**

Surfaces were swabbed using sterile cotton swabs then swirled in 450 ul of SPW in a 1.5 ml tube which was then vortexed for 30 sec.

**Sewage and pond water bacterial specimens.**

Sewage were from the Paul R. Noland Wastewater Treatment Facility, while pond samples were from Clarence Craft Park. One ml of sample in a 1.5 ml tube was centrifuged at 8000 x g for 10 minutes at 4 °C. The pellet was resuspended in 450 ul of SPW.

**NaOH concentration.**

Stocks of 1 M NaOH solutions were prepared from solid (Sigma-Aldrich) in SPW.

### **Comparison of two paramagnetic beads.**

Paramagnetic beads were Omega Mag-Bind RXN Pure Plus, and silica-coated BioChain PureSil beads. Omega Mag-Bind RXN Pure Plus beads are provided in binding buffer. For BioChain PureSil-Silica magnetic beads (Catalog #: L5011010). Bead binding buffer was made from 8 ml isopropanool + 2 ml of 4 M GuSCN (dry), 40 mM TrisCl pH8, 17.6 ml Na<sub>2</sub>EDTA (BioChain Institute Inc., Newark, CA).

### **DNA extraction from bacterial cultures.**

Bacterial cells were acquired from bacterial cultures grown in broth media or on agar plates. The cells were lysed with 100 mM NaOH (pH 12.9). To prepare a cell suspension from the colonies of agar plates, a single colony was suspended in 25 ul of SPW. Then, 10 ul of the overnight broth culture or the cell suspension was mixed with 10 ul of 200 mM NaOH, left for 10 minutes at room temperature (RT), and then diluted with 80 ul of Te.

### **DNA extraction from environmental specimens.**

Different DNA extraction methods were examined: a) Rapid boiling DNA extraction (Holmes & Quigley, 1981; Trkov and Avgustin, 2003) in which the cells are boiled at a temperature of 100°C for 10 minutes inside a PCR machine; b) Sonication for bacterial genomic DNA extraction (Zhang et al., 2005) with rapid boiling, in which the cells were sonicated 10 times, each time for 30 seconds, followed by 3 minute stop interval; c) The glass bead-beating method (Teng et al., 2018), in which the cells were bead-beaten using sterile 0.1–1.5 mm beads in a

mixer; d) The enzymatic extraction method using lysostaphin and lysozyme (Zhao et al., 2012); e) The extraction method using 1-ethyl-3-methylimidazolium acetate (C2minOAc) ) (Martzy et al., 2019) by mixing 81 ul C2mimOAc (95%) with 9 ul Te to the cells; f) Sodium hydroxide (NaOH) lysis followed by neutralization by sodium acetate (Natarajan et al., 2016); and g) NaOH lysis without neutralization by sodium acetate, in which the airborne bacterial cells that were re-suspended in 100 ul of SPW were treated with 11 ul of 1 M NaOH and incubated at RT for 10 minutes. Also, the other environmental samples in 450 ul of SPW were treated with 50 ul of 1 M NaOH and incubated at RT for 10 minutes. The bacterial NaOH lysate solution was then centrifuged at 8000 x g for 10 minutes at 4 °C. One hundred ul of the supernatant was transferred to a new 0.5 ml tube.

#### **Direct dilution method.**

The NaOH lysate was diluted 1:5 with Te, and then 2 ul of the DNA was directly used per 20 ul PCR volume for running PCRs and/or qPCRs (Fidler et al., 2020). The DNA was stored at -20 °C.

#### **Paramagnetic beads DNA purification procedure.**

One hundred ul of NaOH lysates was mixed with either an equal volume of Mag-Bind RXN Pure Plus (Omega Bio-Tek)), or with 95 ul of bead binding buffer and 5 ul of the BioChain PureSil beads, at RT in a 0.5 ml microfuge tube. The suspension was mixed by vortexing for 30 seconds, incubated at RT for 2–5 minutes, and then captured on a magnet stand. The supernatant was pipetted off and discarded. The captured beads were rinsed twice for 1 minute with 200 ul of

70% ethanol in the stand, followed by supernate removal with a pipet. The 0.5 ml tube was then opened while on the magnetic stand for 5 minutes to air dry. The tube was removed from the magnetic stand. Elution solutions were either Te (10 mM TrisCl 0.1 mM EDTA pH7.5), TE (10 mM TrisCl 1 mM EDTA pH7.5), 10 mM Tris pH 8.0, or SPW. The elution solution (30–50 ul) was added to the 0.5 ml tube, vortexed for 30 seconds, incubated at RT for 5 minutes, and then beads were captured on the magnetic stand. The eluate was then transferred to a new 1.5 ml tube and stored at -20 °C. For PCR evaluation, 2 ul of this preparation was used in a 20 ul PCR.

### **Quantification of extracted DNA.**

The NaOH lysate DNA from the air samples was then subjected to quantification using qPCR with 16S primers (8F and 936R) targeting the V1–V5 region of the 16S rDNA gene (Baker et al., 2003; Edwards et al., 1989). Reactions were 20 ul comprising 2 ul of 10x Taq Buffer, 0.2 ul 20 mM dNTPs, 0.1 ul 50 uM primers, 1 ul of 20X EvaGreen® Dye (Biotium, Fremont, CA, USA), and 0.05 ul 80U/ul Taq Polymerase. Reactions were carried out in triplicate in 96 well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The cycles were as follows: initial denaturation at 90 °C for 45 seconds, 5 cycles of denaturation at 90 °C for 15 seconds, annealing at 71.5 °C for 15 seconds, and extension at 72 °C for 60 seconds, followed by 35 cycles with the same parameters and a plate read after each cycle. Then, a High Resolution Melt consisting of 72° C for 140 s, 90 °C for 60 s, and 70 °C for 120 s was performed, followed by monitoring fluorescence during temperature rise from 70 °C to 90 °C with 0.1 °C/ 5 s increments and reading the plate.

## **Statistical analysis.**

Student T-Test was used to compare means of Ct values from qPCR, where significance difference was at  $P < 0.05$ .

## **Results.**

### **Optimization of DNA extraction.**

We evaluated the efficiency of DNA extraction methods, including rapid boiling; sonication; bead-beating; enzyme digestion; and C2minOAc, by qPCR using the bacterial 16S primers (Table 5.1). We have used rapid DNA extraction methods to extract DNA from bacterial culture cells. we examined sodium hydroxide (NaOH) lysis followed by sodium acetate neutralization to extract DNA from the cells. Then, we used NaOH alkaline lysis without sodium acetate neutralization.. The qPCR assays were performed with 1–2 ul of the extracted DNA per 20 ul of PCR volume using 16S primers (Table 5.2). Although the boiling approach was sometimes successful, it was not reproducible and was not effective against some bacterial cultures. Also, no DNA was detected after lysis with NaOH followed by neutralization with sodium acetate (Table 5.3). Using 100 mM NaOH we were able to recover DNA. We were able to extract DNA using 10 mM NaOH with C2mimOAc (Fig. 5.2). However, the results were not repeatable (Table 5.3). We were unable to acquire any 16S rDNA gene amplification using rapid boiling with C2mimOAc (Table 5.3).

Our qPCR with 16S primers (Table 5.2) were effective in identifying extracted DNA using a variety of techniques. Table 5.1 displays the outcomes of our most current alkaline lysis

method for DNA extraction from several different bacterial species obtained from lame birds (Fig. 5.3). No species were resistant to NaOH lysis (Table 5.1).

We added different NaOH concentrations into qPCR reactions. In addition, we adjusted 20 ul PCR reactions to 18 different NaOH concentrations (mM) to investigate the qPCR inhibitory impact of NaOH (Fig. 5.4). We also evaluated four different NaOH concentrations (Fig. 5.5): 25, 50, 75, and 100 mM NaOH in the lysis buffer on  $4.0E+03$  CFU of bacterial culture cells grown in broth. The effect of 100 mM NaOH lysis on pure DNA was tested (Fig. 5.6) by subjecting pure bacterial DNA to 100 mM NaOH versus pure water (SPW). At the final concentration of  $\leq 5.6$  mM NaOH, no inhibition was detected. While, at a concentration of 7.5 and 10 mM NaOH, the amplification was only partially inhibited. Therefore, we did not test any greater NaOH final concentrations in the rest of the experiments. Based on these findings, we found that 2 mM NaOH is the least threatening final NaOH concentration per 20 ul PCR reaction to use.

We examined the impact of NaOH lysis on DNA in which pure bacterial DNA was exposed to 100 mM NaOH versus SPW. We found that the difference was insignificant. The t-test P-value was 0.43, and the average Ct value was around  $12.5 \pm 0.08$  (Fig. 5.6). This indicates that 100 mM NaOH has no destructive effect on DNA in any way besides rendering it single-stranded.

Four different NaOH concentrations; 25, 50, 75, and 100 mM NaOH were used in the lysis buffer on  $4.0E+03$  CFUs of bacterial cells from broth cultures. The cells were resuspended in each NaOH concentration for 10 minutes at RT. We diluted the bacterial NaOH lysates with Te (or water) in a 1:5 ratio, followed by paramagnetic bead DNA purification. The performance of 75 and 100 mM NaOH was marginally increased (Fig. 5.5). We found these NaOH

concentrations in the lysis buffer produced comparable DNA yields. We selected 100 mM NaOH as the reliable concentration of NaOH in the alkaline lyse solution for all subsequent tests. Eventually, we repeated the cell lysis processes 15 times using 100 mM NaOH to learn more about the variation of the DNA yields from each cell type.

We treated water washed bacterial cells from broth cultures with 100 mM NaOH at room temperature (RT) versus 37 °C water baths for incubation. We found no significant difference between these two temperatures. The t-test P-value was 0.11, with the average Ct value around  $17.0 \pm 0.25$  (Fig. 5.7). Therefore, we successfully optimized the incubation temperature to RT, ~20 °C. The room temperature is the simplest and requires no specialized equipment. We recommend that the DNA be incubated at RT for 10 minutes in a 100 mM NaOH solution to cause the least amount of fragmentation. In previous protocols and commercial kits, it was 65 or 94 °C for 30 or 90 minutes, respectively (Kouduka et al., 2011; Morono et al., 2014).

*E. cecorum* 1675 cells were treated with 100 mM NaOH at RT for five different incubation times; 0, 5, 10, 20, and 30 minutes separately in triplicates, and then the bacterial NaOH lysates were purified using paramagnetic beads (Fig. 5.8). We found no significant differences between the incubation times of 0, 5, 20, and 30 minutes, and the t-test P-values were 0.06, 0.33, 0.21, and 0.06, respectively. In the case of the incubation time of 5 minutes, there was a significant difference compared with the 30 minute incubation time, with a t-test P-value of 0.022. However, in the case of the incubation time of 10 minutes, there were significant differences between 10 minutes and the other four incubation times; 0, 5, 20, and 30 minutes, with t-test P-values of 0.021, 0.024, 0.025, and 0.003, respectively. The incubation time of 10 minutes resulted in a lower Ct value of  $25.44 \pm 0.41$ . In the end, we performed the cell lysis procedures 20 times with a 10-minute incubation period.



We tested eight logs of cells from broth cultures, ranging from  $8\text{E}-02$  to  $8\text{E}+05$  CFUs. The washed cells were directly added to 20  $\mu\text{L}$  PCRs without prior treatment with 100 mM NaOH. We found reliable amplifications for  $8\text{E}+02$  to  $8\text{E}+05$  CFUs (Fig. 5.9). We also discovered that using total DNA extracted from  $8.0\text{E}+04$ – $8.0\text{E}+05$  CFUs per 20  $\mu\text{L}$  of PCR results in PCR overload. When we compared this to a similar range of bacterial cells extracted with 100 mM NaOH there was amplification  $1\text{E}+01$  to  $1\text{E}+07$  CFUs. Remarkably, our NaOH technique was able to recover sufficient DNA for qPCR tests from as little as  $1.0\text{E}+01$  CFUs of bacterial cells (Fig. 5.10). Thus, NaOH extraction produced a qPCR amplification even from as few as 10 CFUs. It is worth noting that our air sampling technique allowed us to gather no less than 130 CFUs of bacteria (Table 5.3). The smallest average Ct value was found using  $1.0\text{E}+06$  CFUs to extract DNA using NaOH lyse.

We analyzed the 1:5 dilution to overcome the inhibitory effect caused by NaOH, and/or the cell components (Wang et al., 1993). qPCR experiments were used to evaluate the extracted DNA (Table 5.3-5.4). The qPCR assay was chosen to observe declines in the efficiency of amplification (Wang et al., 2021). Because of the strong inhibitory effect on the amplification process, the higher NaOH concentrations would be inappropriate for our DNA production technique.

To determine the effect of qPCR inhibition, we exposed 20  $\mu\text{L}$  PCR reactions containing 0.25 ng/ $\mu\text{L}$  DNA to 18 different concentrations (mM) of NaOH.

To further purify the NaOH extracts, we used paramagnetic beads, such as Omega Mag-Bind RXN Pure Plus, and BioChain PureSil-Silica beads (BioChain Institute Inc., 2022). We found that the NaOH extraction method would be much more useful if it could be combined with

the paramagnetic bead purification method. To purify the NaOH extract, the paramagnetic beads have been used according to the protocol explained in the methods.

We have compared both Omega Mag-Bind RXN Pure Plus and BioChain PureSil-Silica beads in the purification of bacterial DNA extracted by NaOH. The average Ct values for the BioChain versus the commercially available MagBind beads were  $25.09 \pm 0.30$ , and  $30.12 \pm 0.32$ , respectively (Fig. 5.11). It has been shown that both paramagnetic beads can efficiently bind DNA isolated from various bacterial samples. However, there was a significant difference between the BioChain and the Mag-Bind beads. The t-test P-value was 0.001, suggesting that the BioChain beads were preferred over the Omega Mag-Bind RXN Pure Plus beads. The BioChain beads are cheaper and recover 15–20% more DNA than MagBind beads in the extracted DNA from bacterial colonies.

For the comparison of paramagnetic bead purification versus 1:5 dilution in Te, we tested the effect of the 100 mM NaOH-based technique on 3 Gram-positive and 3 Gram-negative bacteria to investigate the amount of yielded DNA acquired using both methods (Fig. 5.12). The t-test P-values for the average Ct values obtained after bead purification and 1:5 dilution with Gram-positive as compared to Gram-negative bacteria were 0.015 and 0.12, respectively (Wang et al., 2020; Osmundson et al., 2013). The paramagnetic bead purification resulted in a higher amount of DNA, especially in Gram-positive bacteria, with an average Ct value of  $14.63 \pm 0.13$ , whereas the 1:5 dilution yielded an average Ct value of  $16.75 \pm 0.40$  in the same bacterial NaOH extracted DNA. Therefore, after paramagnetic purification, the 1:5 dilution of the bacterial NaOH lysate is the second best, quicker, and cheaper method, especially for Gram-negative bacteria, in which no extra equipment is required. Consequently, we repeated the process of

purifying and diluting the extracted DNA 15 times to better understand the diversity of the recovered DNA yields.

We compared fresh and frozen air-sampled DNA extracted with NaOH from the same air sample, in which no significant difference was found between 1 hour and 3 months post DNA extraction. The t-test P-value was 0.06 (Fig. 5.13). Therefore, we have determined that even after 3 months of storage at -80 °C the extracted DNA was not degraded. As a result, if the DNA extracted with NaOH lyse is not immediately used, it can be safely stored at -20 °C.

We added eight different numbers of CFUs of bacterial culture cells grown in TSB, then washed in SPW, and then added to the PCR reactions: 8.0E-02, 8.0E-01, 8.0E+00, 8.0E+01, 8.0E+02, 8.0E+03, 8.0E+04, and 8.0E+05 CFUs (Fig. 5.9). On the other hand, different number of bacterial cells, *S. agnetis* 908 CFUs, were subjected (Fig. 5.10) to the 100 mM NaOH lysis procedure to extract DNA for qPCR experiments, and then NaOH lysates were diluted (5x) in Te. On the NaOH lysates, we also compared paramagnetic beads (Fig. 5.11), such as Omega Mag-Bind RXN Pure Plus and BioChain PureSil-Silica beads (BioChain Institute Inc., 2022). Moreover, we tested how well paramagnetic beads versus a 1:5 dilution worked on bacterial DNA that was extracted using 100 mM NaOH (Fig. 5.12), using three pure Gram-positive bacteria, *S. agnetis* 908, *S. aureus* 1516, and *E. faecalis* 1558, and three pure Gram-negative bacteria, *E. coli* 1409, *E. fergusonii* 1412, and *Salmonella enterica* 1414, which were all cultured overnight at 37 °C in TSB broth, and used as model organisms.

Two incubation temperatures, room temperature (RT) versus 37 °C, were tested (Fig. 5.7) for the 100 mM NaOH lysis in triplicates. Also, we tested five different incubation times for 100 mM NaOH at RT (Fig. 5.8): 0, 5, 10, 20, and 30 minutes, in triplicates.

Pelleted cells from air samples were treated with 100 mM NaOH to lyse the cells and release genomic DNA into the solution, which was then diluted 1:5 with TE.

We compared the 100 mM NaOH extraction technique to the standard rapid boiling procedure using four different bacterial strains. Among the Gram-positive bacteria, we selected four of the most recalcitrant *Enterococcus* strains: *Enterococcus faecalis* 1558, *E. faecalis* 1582, *E. faecalis* 1570, and *E. cecorum* 1675, which were cultured from BCO lesions in lame broilers from our research farm experiments. Following DNA extraction using both techniques, the samples were subjected to qPCR analysis targeting the 16S rDNA gene. The results (Fig. 5.14) showed that the NaOH alkaline lyse method was superior to the rapid boiling method for 1558 ( $P=0.00012$ ), 1582 ( $P=0.0014$ ), and 1570 ( $P=0.0008$ ) (Fig. 5.14). The recovered DNA using 100 mM NaOH was increased by 30–40%. It is worth mentioning that in the case of the most recalcitrant bacteria, *E. cecorum* 1675, the rapid boil method completely failed to extract any amount of DNA.

We found *E. cecorum* 1675 to be a recalcitrant bacterium. Therefore, we used it as a model bacterium to improve our NaOH method for extracting bacterial DNA. We used 3 plates of 1675 to collect colonies. The plates were very old (2 weeks), old (1 week), and new (48 hours old). We used *E. faecalis* 1558 as a control. *S. agnetis* 908 DNA (0.25 ng) was the positive control. No qPCR signal was obtained from the very old colonies (Fig. 5.15) and the new colonies gave a significantly lower Ct value than colonies from the old plate ( $P=0.002$ ).

We could extract DNA from *E. cecorum* 1675 cells from overnight TSB cultures. Cells from 10  $\mu$ l of the culture were treated with 100 mM NaOH, followed by purification using paramagnetic beads and/or a 1:5 dilution with Ct values presented in Figure 5.16.

We compared qPCR results from fresh 100 mM NaOH bacterial cell extractions to extracts frozen for 3 months from the same air sample and found no significant difference (Fig. 5.13). We tested the 100 mM NaOH extraction on cells from broth cultures of a variety of Gram-negative and Gram-positive bacteria, isolated from bone lesions of lame broilers. After DNA extraction from them, the DNA samples were subjected to qPCR analysis targeting the 16S rDNA gene. PCR products of their 16S rDNA were cleaned using RapidTip and then sent for sequencing. Thus, we have identified these bacteria as; *Staphylococcus agnetis*, *S. aureus*, *S. gallinarum*, *S. lentus*, *S. equorum*, *S. cohnii*, *S. nepalensis*, *S. simulans*, *S. saprophyticus*, *Enterococcus faecalis*, *E. cecorum*, *Escherichia coli*, *E. fergusonii*, and *Salmonella enterica* (Table 5.1). These 10 cultures had previously failed to amplify using the rapid boiling method. Therefore, the NaOH extraction method is more reliable than the rapid boiling method.

We subjected air-sampled bacteria to the NaOH DNA extraction protocol. Using our system for air sampling BCO-related bacteria, we have collected 39 air samples, 22 in spring 2021 and 17 in spring 2022 (Table 5.3-5.4) during BCO trials done on our farm at the University of Arkansas/Poultry Research Facilities (Chapter 6). The 16S Ct values of air samples 15–22 were collected for 50–56 days in the spring of 2021 and the Ct value of positive control was recorded (Fig. 5.17). Positive control (1 ul of 0.25 ng/ul). The Ct values were recorded for the air samples 15–22 as shown in Figure 5.16. It is important to note that some of the original air samples from the spring of 2021 were ineffective owing to the initial debugging of DNA extraction from air-sampled bacterial cells prior to the discovery of our NaOH lyse procedure (Table 5.3). The 16S Ct values of the air samples were obtained during spring 2022 at 21–53 d of age (Fig. 5.17). Throughout the sample, we detected *S. aureus* only four times in the air samples at 25, 27, and 31 d of age. (Table 5.4 & Fig. 5.18). ). The repeatability of DNA extractions

compared using different DNA extraction procedures was compared using air samples (Table 5.3-5.4). The NaOH extraction was the most reliable and reproducible.

We carried out the NaOH DNA extraction procedure on soil-borne bacteria. Several garden soil bacterial specimens were collected on the University of Arkansas Campus. To release the DNA, the soil samples were resuspended in 100 mM NaOH with or without 5% saline. Later, the NaOH extracts were either purified with Mag-bind beads or subjected to a 1:5 dilution with Te. The DNA was then used in qPCR assays. The average Ct values of the soil specimens treated with or without 5% saline and then purified using Mag-bind beads or subjected to 1:5 dilution, and the positive control were recorded (Fig. 5.19). We found a significant difference between samples treated with 5% saline and those that were not treated with saline. The treatments performed without 5% saline recovered more DNA, with a t-test P-value of 0.02. In addition, there was a significant difference between using 100 mM NaOH and a 1:5 dilution. The NaOH extracts subjected to purification using paramagnetic beads recovered more DNA, with a t-test P-value of 0.019. Thus, we were able to extract DNA from the soil bacteria in all of the treatments. The DNA was then put through qPCR experiments, and the results were reliable and repeatable (Smalla et al., 1993; Zhou et al., 1996; Kouduka et al., 2011).

The NaOH extraction was tested for chicken cloacal swabs (Fig. 5.20) collected at the University of Arkansas Poultry Research farm. Extracts were captured with paramagnetic beads or diluted 1:5. The average Ct values are shown in Figure 5.21. The Ct values for paramagnetic purification were a little smaller but not significant ( $P=0.13$ ).

The NaOH extraction was applied to swabs from cheese slices, or bread. Average Ct values are in Figure 5.22. Alternative methods of DNA extraction from foods with high fat and protein content typically in the release of high amounts of PCR inhibitors (Rossen et al., 1992).

We found that DNA could easily be extracted from these samples with no evidence of PCR inhibition.

Swabs from various environmental surfaces were treated with 100 mM NaOH to extract DNA followed by Mag-bind bead capture. Average Ct values for the samples and the positive controls are in Figure 5.21. All samples gave 16S amplification.

Sewage and pond water samples were collected and treated with 100 mM NaOH. Samples were either diluted 1:5 or DNA captured with Mag-bind beads. Then, qPCR assays were conducted on the DNA. From the sewage or pond water specimens of A-C subjected to purification using Mag-bind beads and 1:5 dilutions and the positive control, we obtained average Ct values (Fig. 5.23). We found a high degree of reproducibility with the paramagnetic purification producing lower Ct values than the 1:5 diluted DNAs, but there was no significant difference ( $P=0.31$ ). The Ct values for the sewage water were lower, showing a higher bacterial load than the pond water samples.

Using the NaOH method, we extracted bacterial DNA from several environmental samples. The bacterial samples include air samples; soil-borne bacterial samples; chicken cloacal swabs; food samples; samples of uncontaminated surfaces; and samples of pond water and sewage (Fig. 5.17-23). We compared the average Ct values we obtained from the qPCR assays we performed on these environmental samples. In both experiments, we found the bacterial loads were increasing over time, from day 1 to day 56 (Fig. 5.17-5.23).

We compared the 100 mM NaOH extraction to a number of rapid DNA extraction methods, such as rapid boiling, sonication, bead-beating, enzyme digestion, and C2minOAc. We initially compared NaOH (100 mM) and rapid boiling (Fig. 5.14) using four bacterial strains.

The NaOH method was superior to 10 mM NaOH plus C2mimOAc (Martzy et al., 2019) as shown in Figure 5.2.

## **Discussion.**

The NaOH technique for bacterial DNA extraction does not need complex laboratory equipment, other than 100 mM NaOH and incubation at RT for 10 minutes, hence enhancing molecular clinical detection technologies.

We examined certain factors to enhance the reliability and efficiency of the NaOH DNA extraction procedure. A large portion of the parameters we looked at were based on reports from the literature regarding other microorganisms collected in a variety of different environments.

In the first stage, we looked at the inhibitory impact of different NaOH concentrations on the rest of the molecular diagnostic techniques. Normally, the lysing buffer should be able to facilitate sufficient DNA extraction while also not interfering with the amplification process. We evaluated the tolerable concentration of the NaOH by adding it to the qPCR reactions in different concentrations.

We compared the alkaline lyse method with different rapid methods for DNA extraction from bacterial cells collected from air samples. These included rapid boiling; sonication; bead-beating; enzyme digestion; and C2minOAc.

Thus, the final NaOH protocol was successful 25 times out of 30 times when applied to bacteria that were grown in pure culture. We also successfully repeated this protocol 15, 12, 5, 6, 6, and 6 times to extract bacterial DNA from the air, garden soil, chicken cloacal, food, environmental surfaces, sewage, and pond water, respectively. This technique was tuned to



provide a high degree of reproducibility, the lowest incubation time, and a feasible quantity of easily amplifiable PCR products.

DNA extraction using NaOH is performed in a single tube, reducing the number of pipetting steps and the quantity of Materials required, eventually resulting in a lower environmental impact. In the era of significant environmental pollution produced by poisons and plastic trash, the use of volatile organic solvents in conventional enzymatic procedures and the growing use of packaging products come with DNA extraction kits. The long-term stability of sodium hydroxide (NaOH) at ambient temperature, as well as the shelf-life stability of NaOH upon storage, were both investigated in a few studies. NaOH (10 M) has a storage life of approximately 3 years (Spectrum Chemical, 2021). Furthermore, there are no concerns about using lower concentrations of NaOH since the shelf-life of 100 mM NaOH diluted in Te is shown to be up to 30 days (Kumar & Ram, 2019). This technique does not need the use of organic solvents, potentially hazardous and inhibiting compounds, or other treatments, such as heating, proteinase, and mechanical treatments, incubation in a water bath (Martzky et al., 2019).

One disadvantage of NaOH DNA extraction might be in converting the dsDNA to ssDNA, which cannot be quantified using the traditional quantification methods, such as fluorimetry. This issue can be overcome using qPCR. The NaOH method of DNA extraction has significant importance when extracting DNA from any bacterial species that cannot be enhanced by cultivation, as well as from bacteria collected from the air because this process is supported by the use of silica-coated paramagnetic beads to purify the DNA from the bacterial NaOH lysate (Clark et al., 2015).

The small amount of template DNA required for effective amplification in PCR, along with a high tolerance for NaOH DNA preparations, suggests that extracting enough DNA using

the NaOH method and using it directly for PCR may be feasible. The technique of DNA extraction using NaOH lysis is likely to be more successful because it provides sufficient dilution of the extracted DNA to remove or substantially reduce the impact of the possible PCR inhibitors (Wang et al., 1993). The optimized NaOH technique has the potential to improve knowledge of the microbial community structure of various habitats (Kouduka et al., 2011). We showed that the extracted DNA can be utilized for conventional or quantitative PCR with relative ease, and it may also be useful for other molecular diagnostic approaches such as DNA sequencing applications or DNA hybridization processes.

This method requires substantially less equipment, time and money to extract DNA from bacteria when compared to the typically time-consuming and laborious procedures that are required by conventional enzymatic approaches or commercial kits. The technique would be feasible to execute even in resource-constrained environments, should save a significant amount of time and money while also reducing the risk of cross-contamination. Also, it could be integrated with tools for clinical diagnostics (Garcia et al., 2016). Using bacterial 16S rDNA genes, this method revealed an increase in bacterial DNA diversity (Morono et al., 2014). Therefore, it provides greater analytical coverage of environmental microbial populations than traditional techniques. Ultimately, we empirically found sodium hydroxide (NaOH) as an efficient and reproducible method to extract DNA from bacterial cells.

To summarize, the preparation of genomic DNA from bacteria using 100 mM NaOH followed by purification using paramagnetic beads or a 1:5 dilution in TE offers many benefits over conventional enzymatic techniques and commercial kits. The proposed technique is distinguished by the fact that it requires just one incubation step of ten minutes, as opposed to the many incubation and centrifugation stages required by most conventional methods, which total

two to three hours for the extraction process. As a result, the management of the process utilizing 100 mM NaOH is considerably easier when several extraction runs are performed in parallel, allowing for high sample throughput. There is also no need for a fume hood, centrifuges, or even the use of a heating block or a water bath, since the reaction may be carried out in a standard laboratory setting at room temperature. A single sample preparation using a commercial kit costs \$5.0, making it the most costly of the commonly used techniques. The preparations with 100 mM NaOH are the most affordable, with an estimated cost of \$1.0 per reaction when consumables like pipette tips and reaction tubes are taken into consideration. The fact that the pricing for 100 mM NaOH was obtained from small sample sizes for laboratory scale applications should be highlighted in this context since costs for 100 mM NaOH are anticipated to be much lower than what is produced on a larger scale.

**Table 5.1.** The bacteria were checked using our final protocol

Scientific names	Strain number	Gram	Source
<i>Staphylococcus agnetis</i>	908	Positive	Pure culture
<i>S. aureus</i>	1516	Positive	Pure culture
<i>Enterococcus faecalis</i>	1558	Positive	Pure culture
<i>E. faecalis</i>	1582	Positive	Pure culture
<i>E. faecalis</i>	1570	Positive	Pure culture
<i>E. cecorum</i>	1675	Positive	Pure culture
<i>Staphylococcus gallinarum</i>	1704	Positive	Lame bird*
<i>S. nepalensis</i>	1705	Positive	Lame bird*
<i>S. simulans</i>	1716	Positive	Lame bird*
<i>S. saprophyticus</i>	1717	Positive	Lame bird*
<i>S. aureus</i>	1719	Positive	Lame bird*
<i>S. lentus</i>	1722	Positive	Lame bird*
<i>Staphylococcus equorum</i>	1727	Positive	Lame bird*
<i>S. cohnii</i>	1728	Positive	Lame bird*
<i>Streptococcus agalactiae</i>	1731	Positive	Lame bird*
<i>Escherichia coli</i>	1724	Negative	Lame bird*
<i>E. coli</i>	1409	Negative	Pure culture
<i>E. fergusonii</i>	1412	Negative	Pure culture
<i>Salmonella enterica</i>	1414	Negative	Pure culture

Lame bird\*: Cultures were unknown and isolated from lame broilers.

**Table 5.2.** Primers were used in the qPCR methods to determine the genomic DNA of the bacteria used in this study.

Experiment	Primer's code	Sequence 5' → 3'	Literature.
qPCR targeting 16S	8 F	AGAGTTTGATCCTGGCTCAG	Edwards et al., 1989
	936 R	AATTGACGGGGGCCCCGCAC	Baker et al., 2003

**Table 5.3.** Air specimens and the methods used to directly extract DNA from air samples (1–22) collected during the Spring 2021 BCO experiment.

Sample#	Age (day)	Duration (min)	CFUs/sample	DNA extraction	Primers	DNA
1	17	3; 10	4100; 8825	Rapidboiling	16S (8Fx936R)	Yes*
2	20	15; 30	12205; 21050	Rapidboiling	16S (8Fx936R)	No
3	21	20	4500	Mixer_sterile_beads	16S (8Fx936R)	No
4	27	20	35200	Sonication	16S (8Fx936R)	No
5	29	20	4000	Sonication	16S (8Fx936R)	No
6	35	20	17200	Sonication	16S (8Fx936R)	No
7	42	20	6000	NaOH+sodium acetate	16S (8Fx936R)	No
8	42	20	6600	Alkaline_lyse	16S (8Fx936R)	Yes
9	44	20	20000	Alkaline_lyse	16S (8Fx936R)	Yes
10	44	20	29300	Alkaline_lyse	16S (8Fx936R)	Yes
11	46	20	46400	Rapidboiling	16S (8Fx936R)	No
12	46	20	20000	NaOH+C2mimOAc	16S (8Fx936R)	Yes
13	48	20	16000	C2mimOAc	16S (8Fx936R)	No
14	48	20	20000	Alkaline_lyse	16S (8Fx936R)	Yes
15	50	20	8000	Alkaline_lyse	16S (8Fx936R)	Yes
16	50	20	12000	Alkaline_lyse	16S (8Fx936R)	Yes
17	52	20	9000	Alkaline_lyse	16S (8Fx936R)	Yes
18	52	20	5000	Alkaline_lyse	16S (8Fx936R)	Yes
19	54	20	12200	Alkaline_lyse	16S (8Fx936R)	Yes
20	54	20	12500	Alkaline_lyse	16S (8Fx936R)	Yes
21	56	20	20000	Alkaline_lyse	16S (8Fx936R)	Yes
22	56	20	52900	Alkaline_lyse	16S (8Fx936R)	Yes
campus**	N/A	20	130	Alkaline_lyse	16S (8Fx936R)	Yes

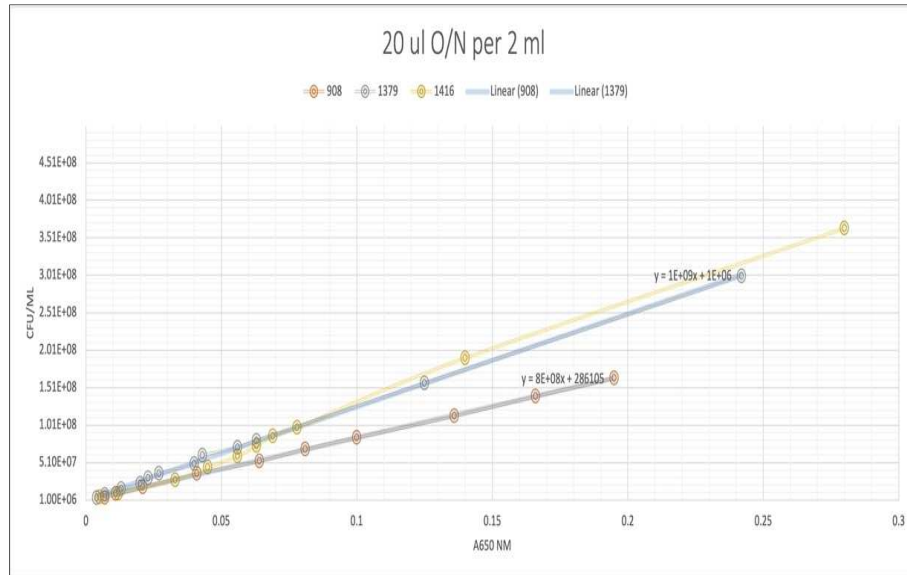
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\*: We obtained bacterial DNA, but the DNA extraction was not repeatable using Rapidboiling DNA extraction. \*\*: University of Arkansas campus.

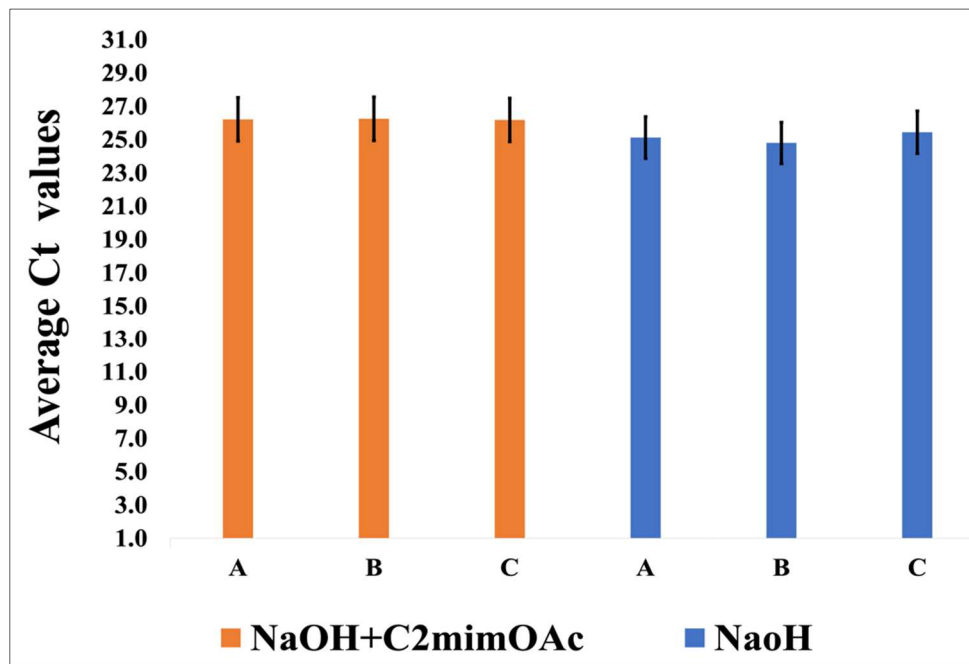
Yes: DNA was obtained. No: DNA was not obtained.

**Table 5.4.** Air specimens and the methods used to directly extract DNA from air samples (A–Q) collected during the Spring 2022 BCO experiment, along with the average Ct values obtained from running qPCRs. N/A: CFUs per air sample were not monitored anymore.

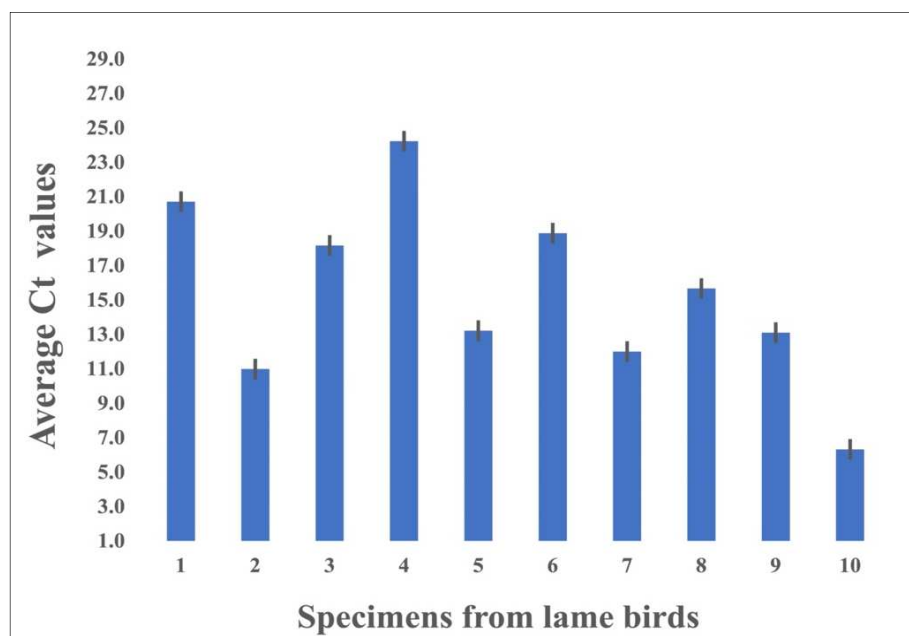
<b>Samples</b>	<b>Age (day)</b>	<b>Duration (min)</b>	<b>CFUs/sample</b>	<b>DNA extraction</b>	<b>Primers</b>	<b>16S Average Ct</b>
1	21	20	340	Alkaline lyse	16S (8Fx936R)	22.3
2	21	20	1520	Alkaline lyse	16S (8Fx936R)	20.5
3	23	20	1260	Alkaline lyse	16S (8Fx936R)	19.5
4	23	20	2010	Alkaline lyse	16S (8Fx936R)	18.3
5	25	20	2080	Alkaline lyse	16S (8Fx936R)	16.0
6	25	20	3640	Alkaline lyse	16S (8Fx936R)	17.4
7	27	20	4580	Alkaline lyse	16S (8Fx936R)	20.1
8	27	20	3650	Alkaline lyse	16S (8Fx936R)	19.3
9	37	20	11200	Alkaline lyse	16S (8Fx936R)	16.0
10	37	20	12800	Alkaline lyse	16S (8Fx936R)	15.8
11	43	20	N/A	Alkaline lyse	16S (8Fx936R)	15.9
12	43	20	N/A	Alkaline lyse	16S (8Fx936R)	16.6
13	43	20	N/A	Alkaline lyse	16S (8Fx936R)	16.0
14	49	20	N/A	Alkaline lyse	16S (8Fx936R)	25.8
15	49	20	N/A	Alkaline lyse	16S (8Fx936R)	24.3
16	53	20	N/A	Alkaline lyse	16S (8Fx936R)	21.1
17	53	20	N/A	Alkaline lyse	16S (8Fx936R)	21.1



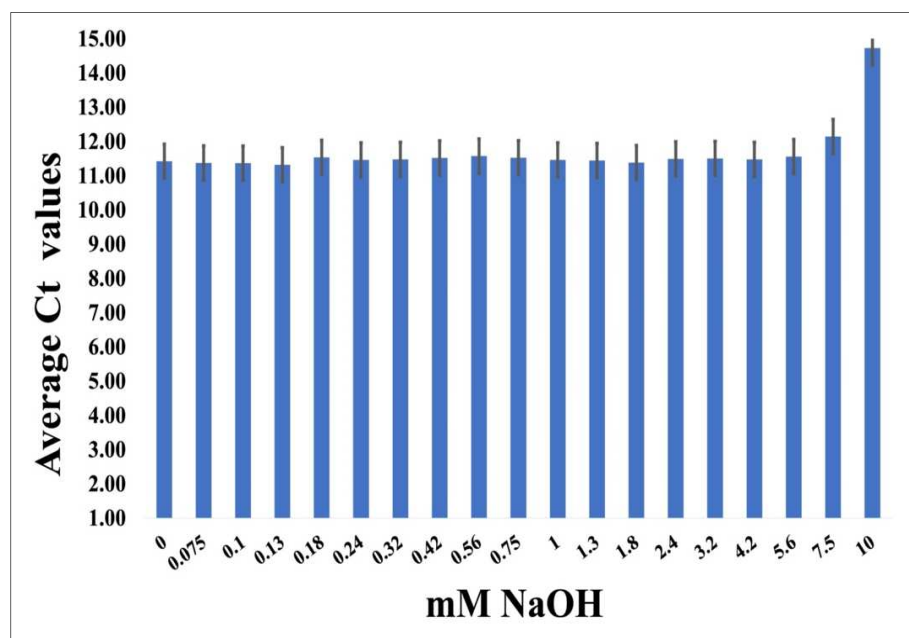
**Figure 5.1.** Turbidity cell density chart for 3 bacteria including *S. agnetis* 908.



**Figure 5.2.** Comparison of 10 mM NaOH+C2mimOAc to 100 mM NaOH for DNA extraction for PCR. A–C are separated samples. Error bars are SEM.

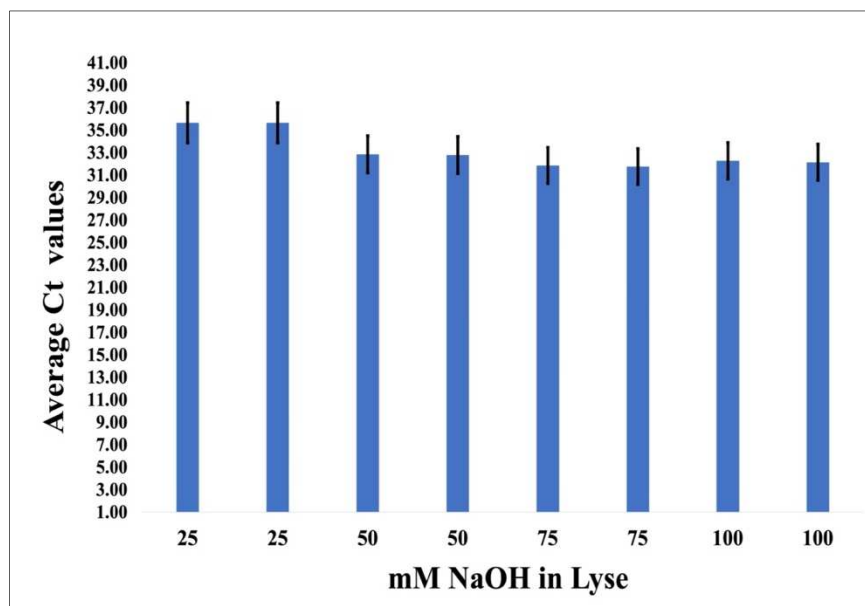


**Figure 5.3.** Extracted DNA from lame birds' bones sampled bacteria and the average Ct. 1-10 are separated bacterial DNA. Error bars are SEM (standard error of the mean).

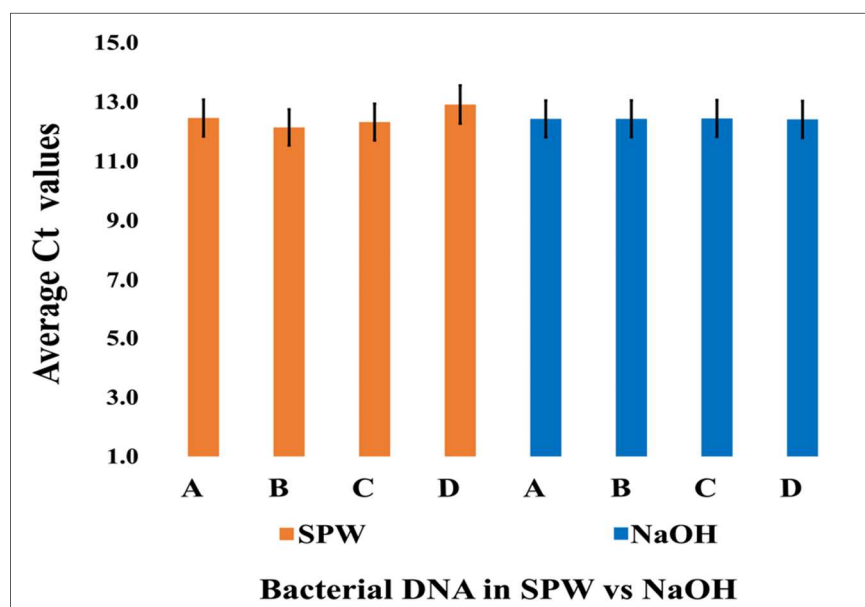


**Figure 5.4.** Different final NaOH concentrations (mM) in PCR, and the average Ct. Error bars are SEM.

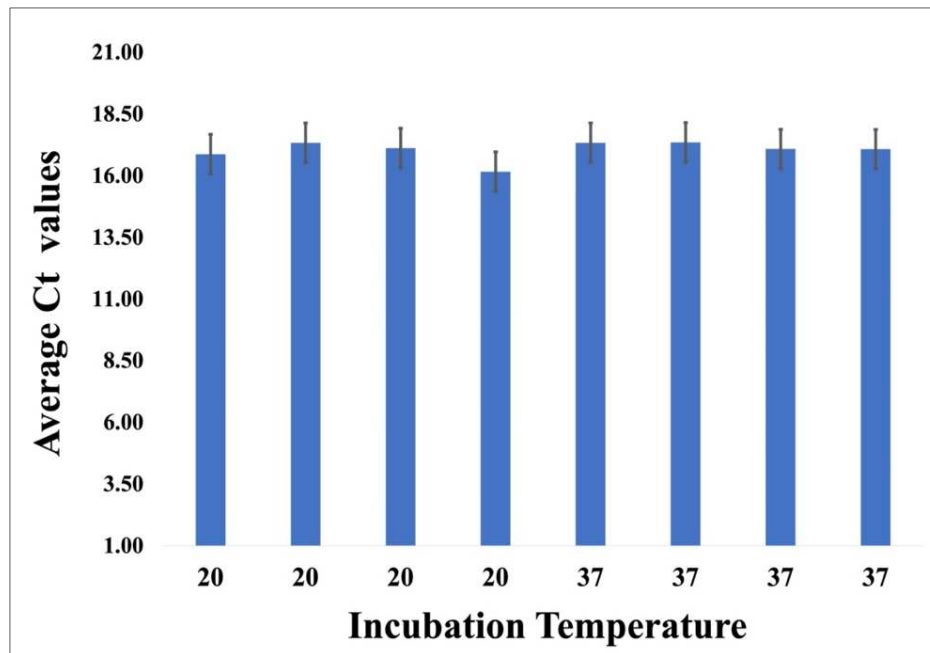




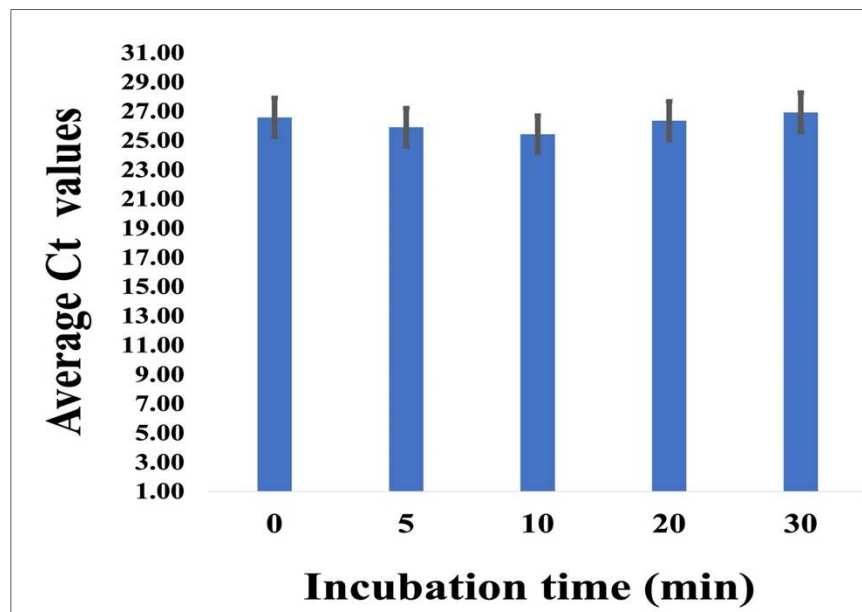
**Figure 5.5.** Different NaOH concentrations (mM) in lyse buffer and the average Ct. Error bars are SEM.



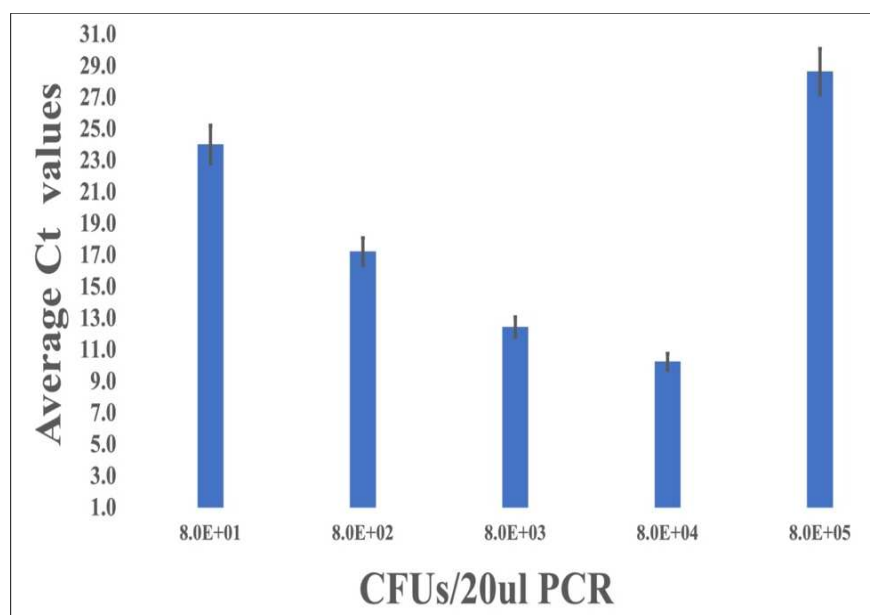
**Figure 5.6.** Pure DNA in SPW versus NaOH lysing buffer, and the average Ct. A-D are separated DNA samples. Error bars are SEM.



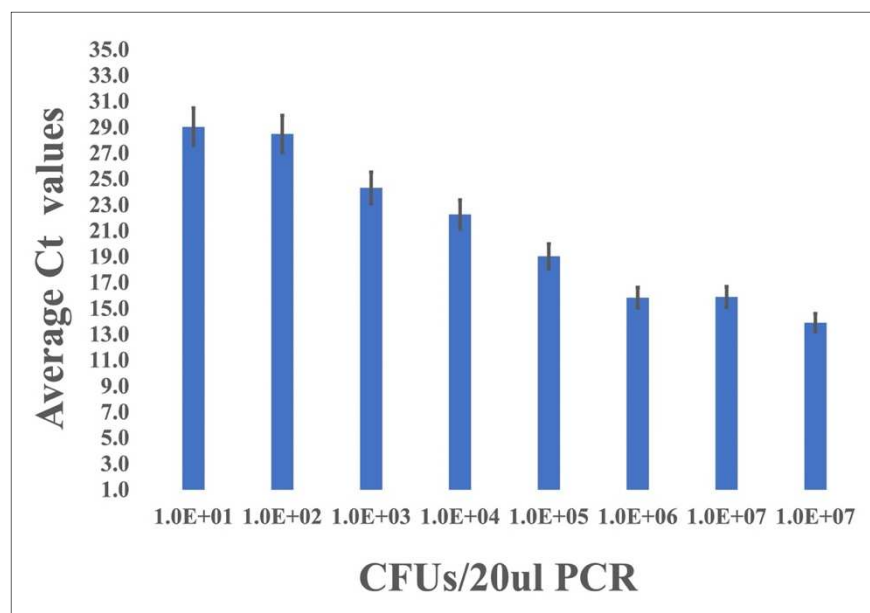
**Figure 5.7.** Different incubation temperatures (°C) for lysing buffer and the average Ct. Error bars are SEM.



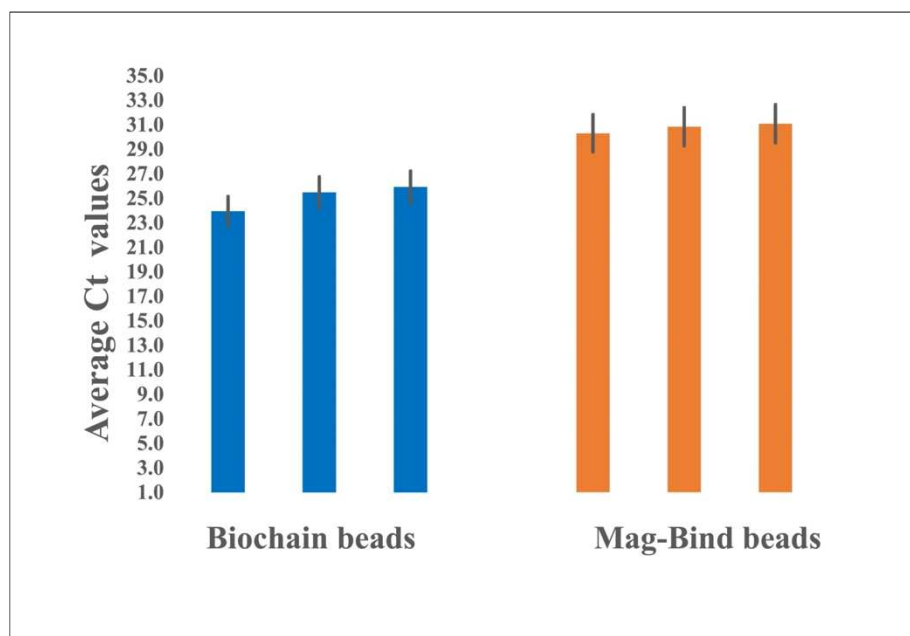
**Figure 5.8.** Different incubation times, and the average Ct. Error bars are SEM.



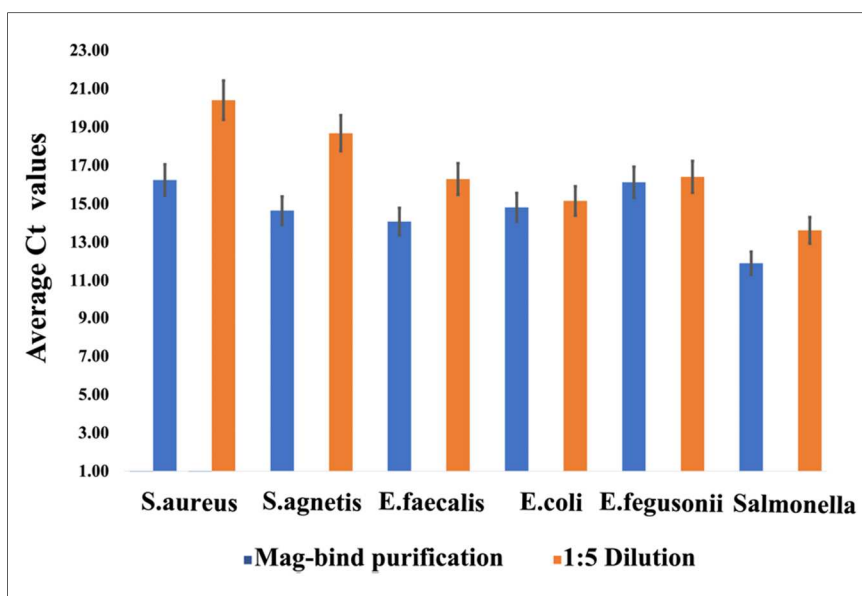
**Figure 5.9.** Different numbers of CFUs per 20 ul PCR reactions, and the average Ct. Error bars are SEM.



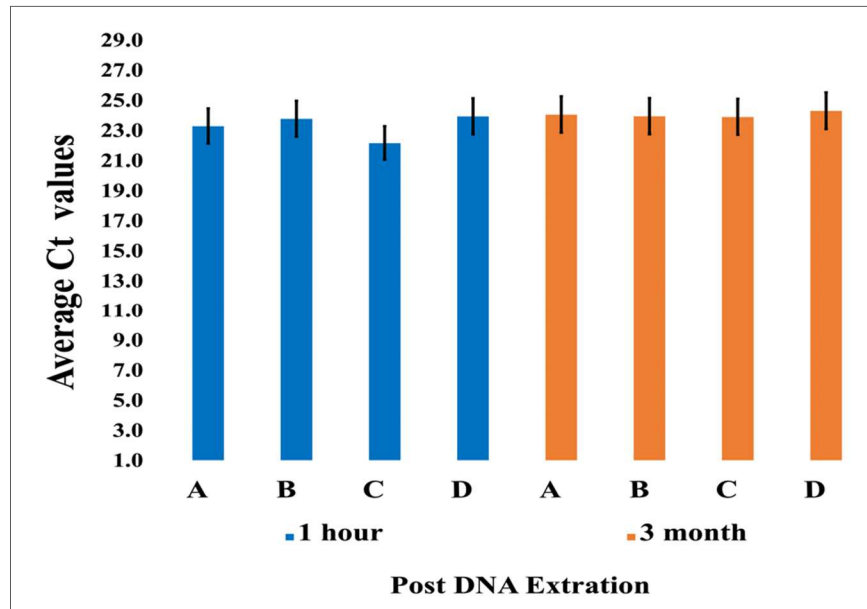
**Figure 5.10.** Different numbers of *S. agnetis* (908) CFUs per 20 ul PCR reactions, and the average Ct. Error bars are SEM.



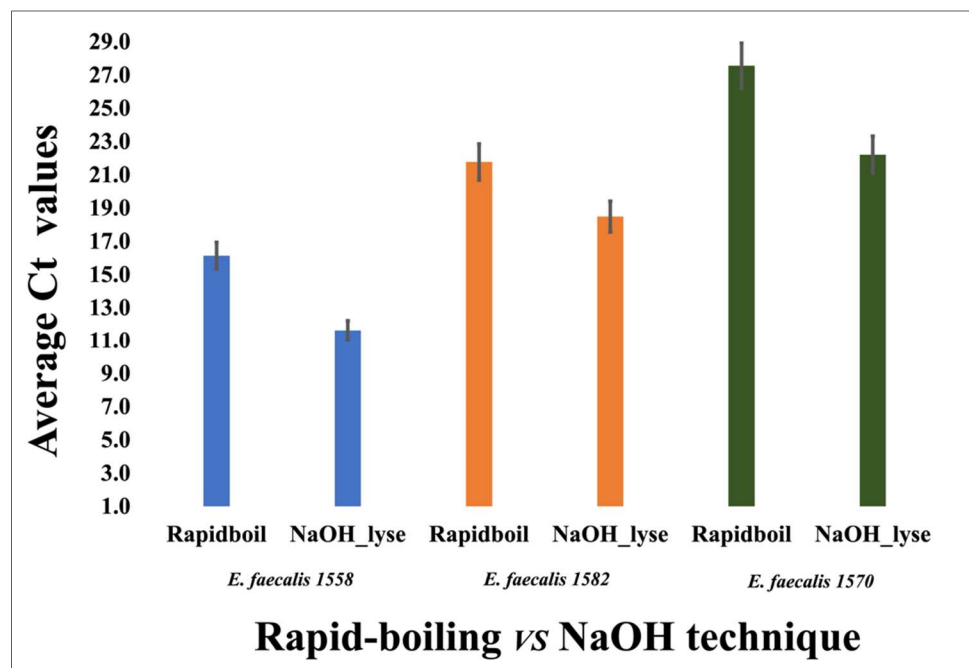
**Figure 5.11.** Different beads (Biochain vs Mag-Bind), and the average Ct. Error bars are SEM.



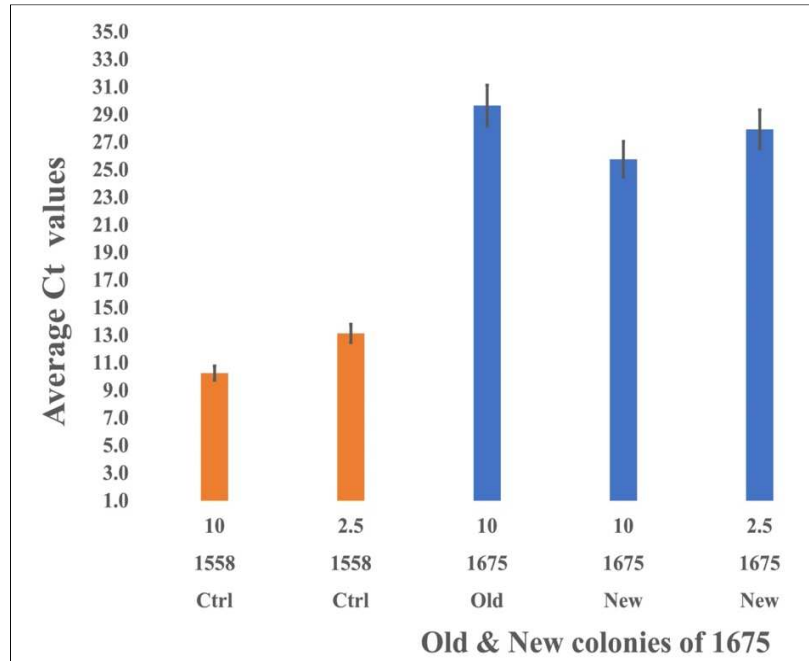
**Figure 5.12.** Paramagnetic DNA purification versus dilution of NaOH DNA extract, and the average Ct. Error bars are SEM.



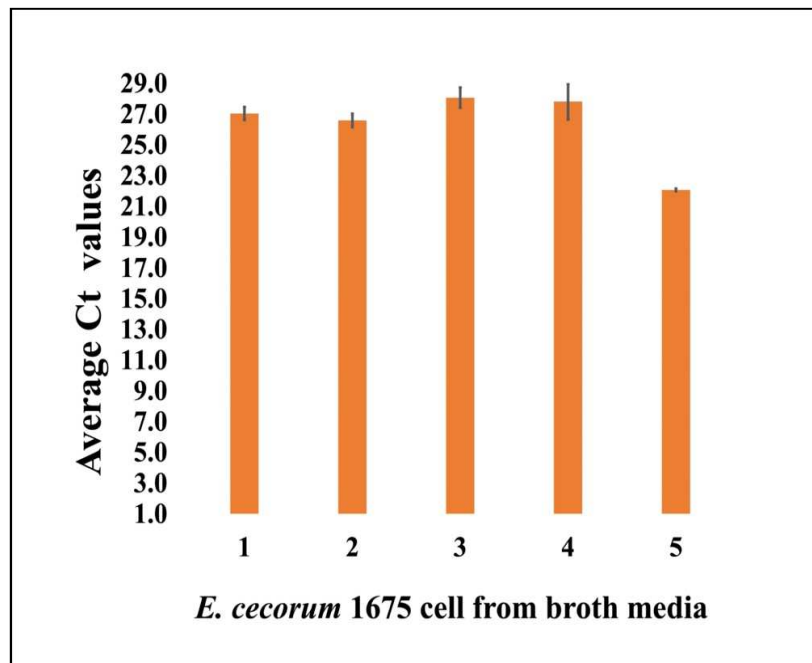
**Figure 5.13.** Times post DNA extraction, and the average Ct. A-D are separated DNA samples. Error bars are SEM.



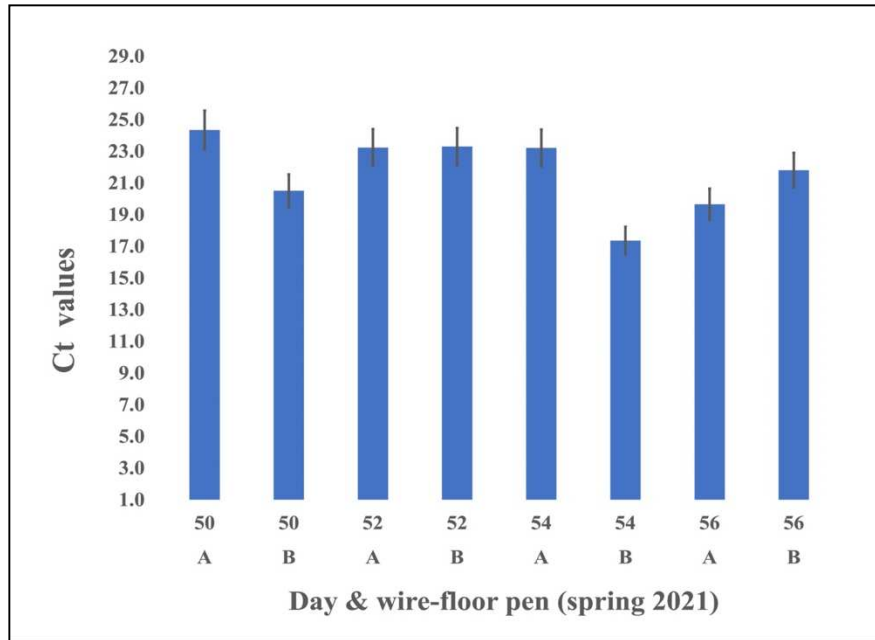
**Figure 5.14.** Extracting DNA from 4 recalcitrant bacterial strains using the Rapid Boil method, versus NaOH lyse, and the average Ct. Error bars are SEM.



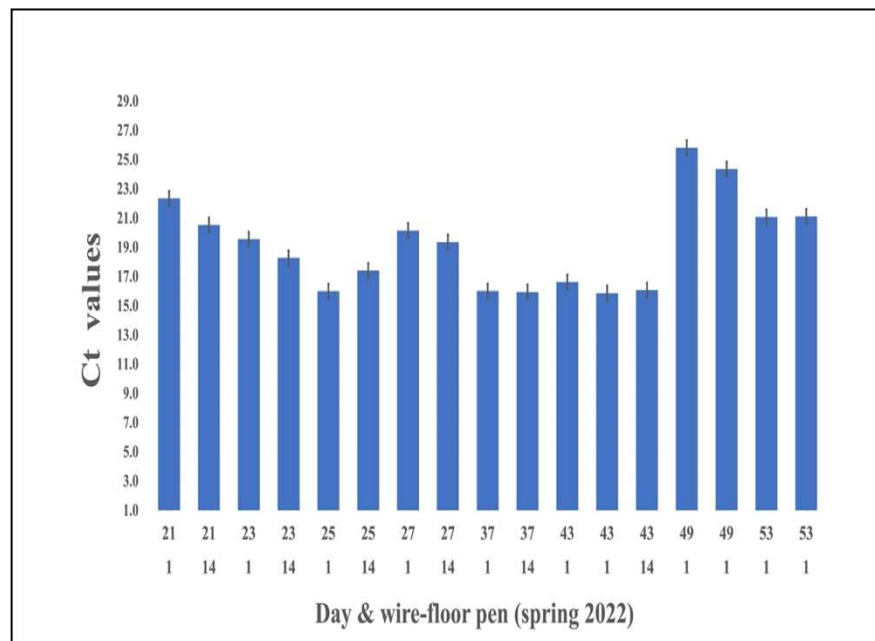
**Figure 5.15.** Old, and new *E. cecorum* 1675 colonies, and the average Ct. Error bars are SEM. 1558 is *E. faecalis* used as control.



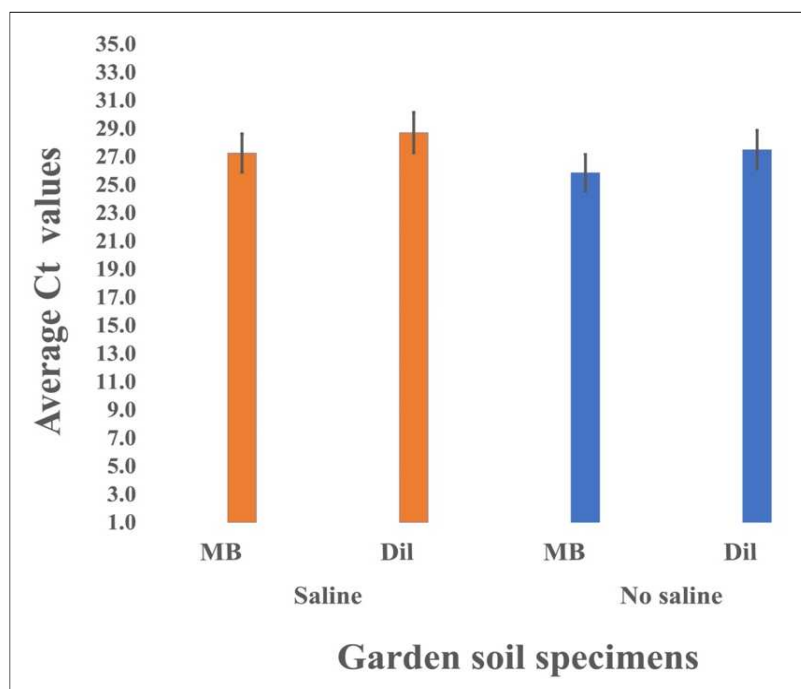
**Figure 5.16.** *E. cecorum* 1675 cell from broth media, and the average Ct. 1-5 are separated samples. Error bars are SEM.



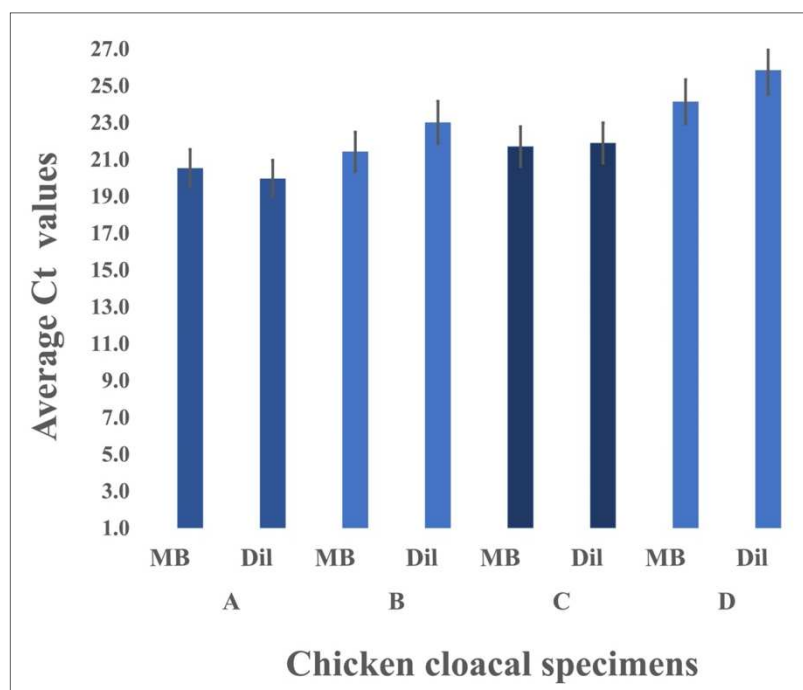
**Figure 5.17.** DNAs extracted directly from air samples collected in spring 2021 using our air sampling system in the wire floor pen A and B, with average Ct values using bacterial 16S primers. Error bars are SEM.



**Figure 5.18.** DNAs extracted directly from air samples collected in spring 2022 using our air sampling system around the wire floor pen 1 and 14, with average Ct values using bacterial 16S primers, 21- 53 d are the day of sampling, Error bars are SEM.

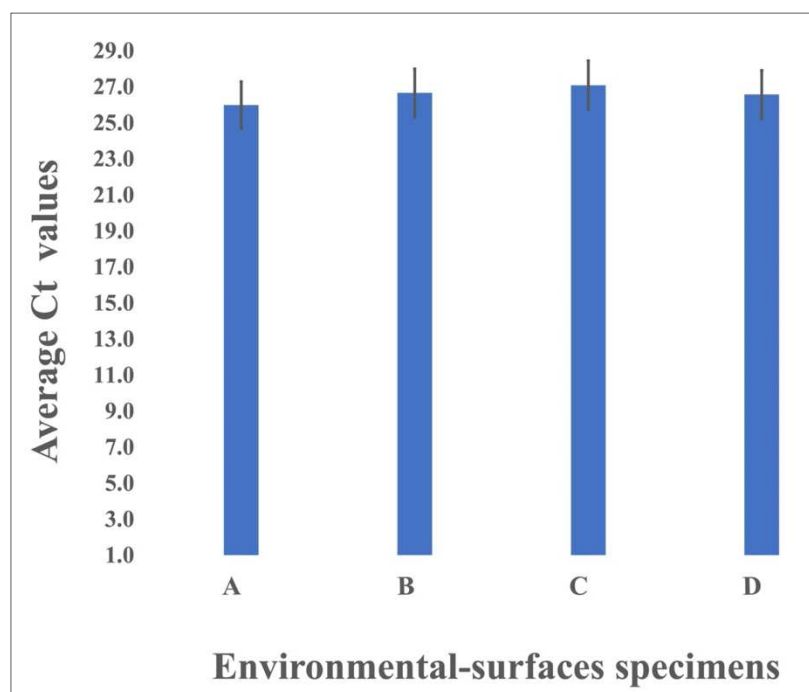


**Figure 5.19.** Extracted DNA from garden soil sampled bacteria, and the average Ct. MB = Mag-bind bead purification; Dil = 1:5 dilution in Te. Error bars are SEM.

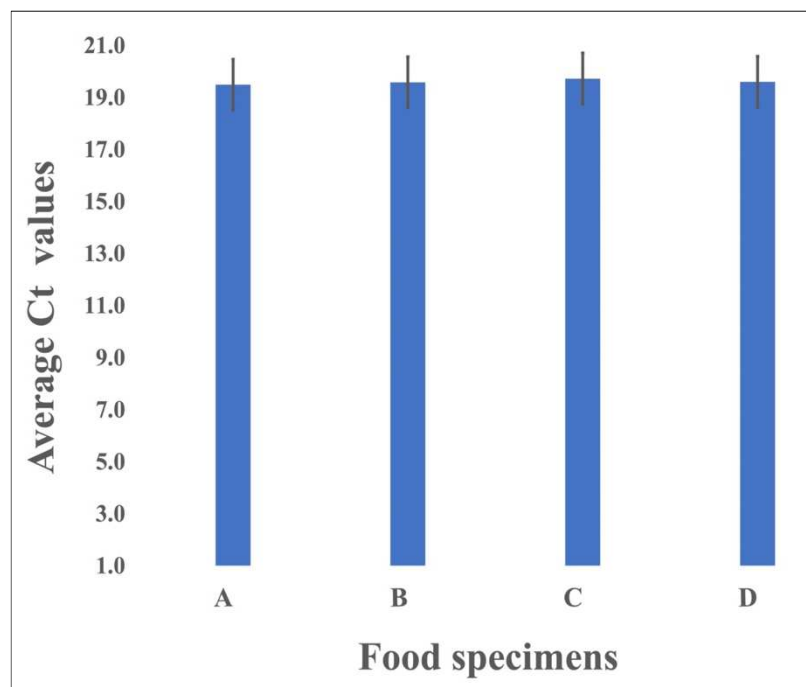


**Figure 5.20.** Extracted DNA from chicken cloacal sampled bacteria, and the average Ct. A-D are separated samples. MB = Mag-bind bead purification; Dil = 1:5 dilution in Te. Error bars are SEM.

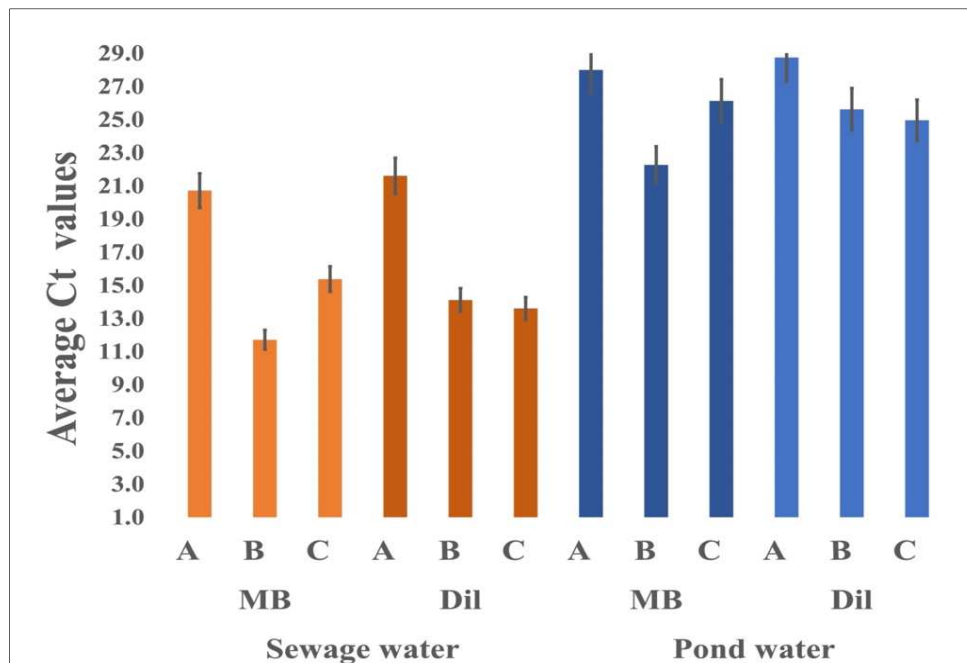




**Figure 5.21.** Extracted DNA from environmental-surfaces samples bacteria, and the average Ct. A-D are separated samples. Error bars are SEM.



**Figure 5.22.** Extracted DNA from food samples bacteria, and the average Ct. A-D are separated samples. Error bars are SEM.



**Figure 5.23.** Extracted DNA from sewage and pond water samples bacteria, and the average Ct. MB = Mag-bind bead purification; Dil = 1:5 dilution in Te. A-C are separated samples. Error bars are SEM.

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## **CHAPTER 6**

**A method for quantifying agricultural pathogen levels in air samples.**



## Chapter 6: A method for quantifying agricultural pathogen levels in air samples.

### Abstract.

This study presents an efficient sampling system for airborne bacterial pathogens. Air sample collection utilized an impinger and portable vacuum pump to collect microorganisms from the air within poultry barns. The purpose was for rapid screening of environmental air samples for agriculturally relevant pathogenic bacteria. A single sample has sufficient cells for screening for multiple species, enabling the identification of a wide diversity of harmful bacteria from each air sample. The test environment was broilers raised on wire-flooring to induce bacterial chondronecrosis with osteomyelitis (BCO). Weekly air samples were obtained for the present experiment on the UA Poultry Research Farm during a BCO induction trial. Agar plates were used for direct collection of air samples. Air was filtered through sterile saline, brought back to the laboratory, bacteria were pelleted by centrifugation, and resuspended in H<sub>2</sub>O. An aliquot was directly plated and the remainder lysed with 100 mM NaOH. The DNA was directly captured using magnetic beads. Staphylococcal species detected by qPCR included *S. lentus*, *S. cohnii*, and *S. aureus*, in air samples taken 17 to 25 days. *S. aureus* and three other BCO-related pathogens were detected in air samples collected from the naturally infected flocks. Our DNA sampling technique is much quicker, simpler, and less expensive than current culture-based approaches. Using the air sampler, we could collect  $13,062 \pm 7,600$  CFUs during a 20 minute sample period. The sampler is manually operated to assure its applicability for use in monitoring airborne pathogens in equipment-limited environments. It is expected that the system could be easily transferred to different agricultural systems and even into human medical systems, to screen for airborne pathogens.

## **Introduction.**

Lameness is a serious animal welfare issue in the poultry industry. A national survey in the US estimated that leg problems cost the broiler industry between 80 and 120 million US dollars annually (Morris, 1993). Lameness caused by bacterial chondronecrosis with osteomyelitis (BCO) in broilers results from a variety of bacterial species infecting the proximal growth plates of the tibiae and femora, and T4 vertebrae (Al-Rubaye et al., 2017; McNamee et al., 2000). BCO epidemics in commercial broiler operations involve several additional bacterial species (Ekesi et al., 2021; Shwani et al., 2020). The studies of Wideman et al. demonstrated that the growth of young broilers on raised wire flooring induces lameness in broilers at a high rate (Wideman et al., 2012, 2013, 2014; Wideman and Prisby, 2013). In this model, lameness is mostly the result of BCO (Wideman et al., 2012, 2013, 2014; Wideman and Prisby, 2013; Wideman, 2016). Al-Rubaye et al. (2015) showed that *Staphylococcus agnetis* was the most common isolate from BCO lesions and blood from lame birds using the wire floor model on the research farm of the University of Arkansas (Wideman, 2016). Al-Rubaye et al. (2017) proved that the administration of these isolates in drinking water increases the incidence of lameness even for birds raised on litter flooring. The infection could also be spread through the air within the same facility (Wideman, 2016; Ekesi, 2020).

Microbial contamination of the air can be a significant risk for animal and human health, especially in the food processing industry (Ellerbroek, 1997; Konieczny et al., 2016). Annually, 420,000 die due to contaminated food, and each year, over \$110 billion is lost in medical care and lost productivity (WHO, 2020; Lues et al., 2007). For over a century, scientists have been studying the spread of disease via the air (Nardell, 2014). However, the makeup of microbial

communities in the atmosphere is still not completely understood (Polymenakou, 2012). Many well-known zoonotic pathogens have been identified in air samples taken from broiler barns, such as *Salmonella* (Kwon et al., 2011; Adell et al., 2014), *S. aureus* (Frieze et al., 2013), *Campylobacter* (Ahmed et al., 2013), *Enterococcus*, and *Enterobacter* (Sanz et al., 2021).

Rapid, cheap, and accurate identification of aerosolized pathogens in housing barns throughout the production phase is critical to implementation of preventive measures. For instance, there are methods for sampling air for specific diseases that use expensive air samplers, such as the CI-95A microbial air sampler, which catches bacteria on membranes. The membranes are transferred to microbiological medium to promote colony formation, and the colonies must then be further processed in order to determine the species (Climet Instructions Company). Other air samplers include the MSP vacuum pump for impactor MOUDI model 100S4 (Stewart et al., 2020), P Trak (Ultrafine Particle Counter, model 8525, TSI), Optical Particle Sizers (OPS, model 3330, TSI), NanoScan SMPS (model 3910, TSI) (Chatoutsidou et al., 2021), and AeroCollect (Andersen et al., 2022). A method for identifying bacterial species using 16S rDNA has been presented (Al-Rubaye et al., 2015; 2017; Ekesi, 2021). Using culture plates for air sampling, Ekesi (2021) found *Staphylococcus cohnii* and *S. lentus* in the air (Ekesi, 2021). These techniques are costly, time-consuming, and can only screen a few colonies per sample, which is insufficient for most applications.

In the current work, we investigated using a standard impinger and a portable pipet pump to sample bacteria from the air during a BCO challenge experiment. We have perfected a system for rapid lysis of bacteria in the sample and DNA capture for subsequent qPCR to measure the overall bacterial load in a mixture as well as to determine the frequency of certain pathogenic species present in that mixture. With this technique, we can look at millions of bacteria for

uncommon diseases in a complicated combination for only a few dollars per sample. Total bacterial load is measured using universal PCR primers for the highly conserved bacterial 16S rDNA gene (Baker et al., 2003), while species specific primers to the plasmin and fibronectin-binding protein A (pfbA) gene measured pathogen load. This system is directly applicable to identifying agriculturally relevant pathogenic microorganisms from air samples and is easily transferable to any other agricultural or medical situation.

## **Materials and Methods.**

### **Bacterial media.**

Media were CHROMagar Orientation (CO) and CHROMagar *Staphylococcus* (CS) (DRG International, Inc., Springfield, NJ), and Tryptic Soy Broth (Difco Laboratories, Franklin Lakes, NJ). Media were prepared according to the manufacturer's specifications.

### **Air sampling instrument.**

The air sampling instrument consists of a manually operated portable pipet pump (Integra Biosciences) attached to a 30-ml sterile glass impinger (Chemglass Life Sciences LLC, Vineland, NJ) containing 20 ml of sterile 0.9% saline solution. Samples were acquired while walking about the barn for 20 minutes with the instrument roughly 30 cm above the ground. The air sampler flow rate was approximately 0.8 liters per minute (Integra Biosciences).

## **Experimental protocols for the identification of *Staphylococcus* species.**

### **Site of sample collection.**

Air sampling was performed during BCO challenge trials at the University of Arkansas Poultry Research farm. Animal protocols were approved by the University Institutional Animal Care and Use Committee (21085 and 21144). The source of the infection was birds raised on suspended wire flooring, and the infection is transmitted through the air to birds on litter flooring (Wideman, 2016). For the two wire-floor pens, 22 and 17 air samples were collected during the BCO experiments carried out in spring 2021 and spring 2022, respectively (Tables 1–2).

### **Air sample collection using the air sampling system.**

Air samples for the wire-floor pens were collected during different days of age of the birds used in the BCO experiments in spring 2021 and spring 2022, respectively (Table 6.1–6.2). Following the collection operation, the impinger contents were transferred to sterile, 50-ml conical centrifuge tubes (Corning, Sigma-Aldrich, USA). The samples were kept at room temperature and transported to the laboratory.

### **DNA extraction and purification procedures.**

The samples were centrifuged at 5000 x g for 10 minutes at 4°C using a Beckman Coulter, Inc. swinging bucket rotor (SX 4400). The supernatant was decanted, the pellets were resuspended in 1 ml of autoclaved sterile pure water (SPW), and transferred to 1.5 ml tubes. Viability counts of the air sampled bacteria were performed by plating 100 ul on CO plates and incubating them at 37°C overnight. And then, the remainder of the suspension was pelleted at 8000 x g for 10

minutes at 4°C. The supernatant was discarded, the pellet resuspended in a volume of 100 µl SPW, and transferred into a 0.5 ml microfuge tube. The suspension was brought to 100 mM NaOH using 1 M NaOH, followed by a 30-second vortex, then incubated at room temperature (RT) for 10 minutes. The NaOH-extracted DNA was captured using an equal volume of MagBind RXN Pure Plus (Omega Biotek, Norcross, GA). The suspension was vortexed for 30 seconds, then incubated at room temperature for 2–5 minutes before being collected on a magnetic stand. The supernatant was discarded. The collected beads were washed twice for 1 minute in the stand with 200 µl of 70% ethanol. The tube was removed from the magnetic stand and the beads were air-dried by opening the 0.5 ml tube for 2–5 minutes. The beads were eluted by adding 50–100 µl Te, vortexed for 30 seconds, incubated at room temperature for 5 minutes, and the beads were captured on a magnetic stand. The eluate was transferred to a new 1.5 ml tube, labeled, and stored at –20°C.

For comparison purposes other DNA extraction methods were rapid boiling DNA extraction (Holmes & Quigley, 1981; Trkov and Avgustin, 2003) by boiling the cells at 100 °C for 10 minutes in a PCR machine; sonication for bacterial genomic DNA extraction (Zhang et al., 2005) with rapid boiling, in which the cells were sonicated 10 times, each time for 30 seconds, followed by 3 minute stop intervals; bead-beating method (Teng et al., 2018), in which the cells were bead-beaten using sterile 0.1–1.5 mm beads in a mixer; enzymatic extraction using lysostaphin and lysozyme (Zhao et al., 2012); extraction with 1-ethyl-3-methylimidazolium acetate (C2mimOAc) (Martzy et al., 2019) by mixing 81 µl C2mimOAc (95%) plus 9 µl Te to the cells; and sodium hydroxide (NaOH) lysis followed by neutralization by sodium acetate (Natarajan et al., 2016).

### **Quantification of extracted DNA.**

DNA from extractions were quantified using qPCR with 16S primers (8F and 936R) targeting the V1–V5 region of the 16S rDNA gene (Baker et al., 2003; Edwards et al., 1989). Additionally, the *Staphylococcus* species associated with BCO were quantified using species-specific primers that target the *pfbA* gene (Table 6.3; Chapter 4). Reactions were 20  $\mu$ l comprising 2  $\mu$ l of 10x Taq Buffer, 0.2  $\mu$ l 20 mM dNTPs, 0.1  $\mu$ l 50  $\mu$ M primers, 1  $\mu$ l of 20X EvaGreen® Dye (Biotium, Fremont, CA, USA), and 0.05  $\mu$ l 80U/ $\mu$ l Taq Polymerase. Reactions were carried out in triplicate in 96 well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The cycles were as follows: initial denaturation at 90 °C for 45 seconds, 5 cycles of denaturation at 90 °C for 15 seconds, annealing at 71.5 °C for 15 seconds, and extension at 72 °C for 60 seconds, followed by 35 cycles with the same parameters and a plate read after each cycle. Then, a High Resolution Melt was performed consisting of 72° C for 140 s, 90 °C for 60 s, and 70 °C for 120 s, temperature increase from 70 °C to 90 °C with 0.1 °C steps in 5 s increments with plate read.

### **Sequencing PCR products.**

Representative PCR products were cleaned using RapidTip® (Chiral Technologies, West Chester, PA, USA), and submitted for capillary sequencing (Eurofins Genomics LLC, Louisville, KY). Clustal Omega in MegAlignPro (DNASStar) was used to create neighbor-joining phylogenetic trees with the 16S sequences.

### **Air sample evaluation using culture plates.**

CO agar plates were used to survey airborne bacteria in the barn. The plates were opened and waved above and around the wire-floor pens for 1 minute. After overnight incubation at 37 °C, colonies were counted and yellow or white colonies were streaked onto CS agar plates using sterile toothpicks. Plates were incubated at 37°C overnight, and probable species determined from color on CO and CS (Fig. 6.1).

### **Sequencing and assembly of the air sampled *Staphylococcus aureus* genomes.**

Pink colonies on the CS plates (Fig. 6.1, Table 6.4) were re-streaked for individual colonies. Genomic DNA was extracted using the phenol-chloroform detailed method described by Dyer & Iandolo (1983), and submitted for PE2x150 sequencing at MiGS (Pittsburgh, PA). The MiGS reads were assembled with the Unicycler ver. 0.4.7 pipeline on the PATRIC website (<https://www.patricbrc.org/>).

## **Results.**

### **Collection system for air samples.**

For the purpose of collecting air samples of agriculturally relevant airborne microorganisms, we assembled an efficient, relatively inexpensive, portable, air sampling system. The system consists of a rechargeable, portable pipet pump and a sterile glass impinger containing saline (Fig. 6.2). Air samples are collected by operating the pipet pump which pulls air through the saline solution. We investigated impingers with either the standard nozzle or



sintered glass flow tube. Air samples were collected while walking in the animal housing while holding the system approximately 1 meter above ground. We also confirmed that there are a number of inexpensive air pumps that are capable of serving as alternatives to the portable pipet pump. These air pumps work on direct current (DC) power (4-6 V) with power supplied by rechargeable 5 VDC battery packs (Data not shown).

To optimize the best period for collecting air samples, different durations of air sampling (3, 10, 15, 20, and 30 minutes) were examined to determine the total CFUs collected from plating 100  $\mu$ l of the 1-ml cell suspensions on agar plates as shown in Figure 6.3. In 20 minutes, an average of  $13,062 \pm 7,600$  CFUs per air sample is sufficient for direct DNA extraction and rapid qPCRs (Andersen et al., 2022). This finding suggests that enough genomic DNA to run the qPCR experiments could be easily obtained in 20 to 30 minutes.

### **DNA extraction system.**

For extracting DNA directly from bacteria collected from air samples, we tested a variety of techniques (Table 6.1). These include: a) rapid boiling; b) sonication; c) glass bead-beating; d) enzyme digestion of cell walls; e) hydrophilic ionic liquid; f) NaOH lysis with sodium acetate neutralization, and g) NaOH lysis followed by 1:5 dilution in Te. The extracts were then assessed using qPCR for 16S rDNA. While the rapid boiling technique was effective at times, it varied in repeatability. The other DNA extraction procedures did not yield qPCR signals from the air samples (Table 6.1). As for other environmental samples (Chapter 5) lysis in 100 mM NaOH without neutralization was sufficient and repeatable, even when the air sample contained few bacterial cells (e.g., 10 CFUs). The least number of bacterial cells we could collect using our air sampling system was 130 CFUs (Table 6.1). In our investigation, we found that the NaOH

technique for DNA extraction worked efficiently, and when the DNA was used in qPCR assays, it was very reliable and repeatable.

### **Collection of air samples during BCO experiments.**

The air sampling system was then used to examine the airborne bacterial load progression during an eight week BCO challenge experiments at the University of Arkansas Poultry Research Farm. Twenty minute air samples were taken once or twice a week during the course of two experiments. We collected 22 air samples in spring 2021 and 17 in spring 2022 (Table 6.1-6.2). For the first experiment samples were collected when the birds were 17, 20, 21, 27, 29, 35, 42, 44, 46, 48, 50, 52, 54, and 56 d of age, versus 21, 23, 25, 27, 37, 43, 49, and 53 d during the second experiment. Corresponding culture-based samples (plate waving) were collected from above and around the same pens. The bacterial loads were estimated using the average Ct values obtained from qPCR with 16S primers (Baker et al., 2003). We used the  $\Delta\Delta C_t$  method (Livak & Schmittgen, 2001) to calculate the fold change in bacterial load at different days during the BCO challenge experiments (Table 6.1–6.2). Over time in both BCO experiments, the 16S signal goes up (Fig. 6.4–6.6). Species specific PCR primers for the *pfbA* gene were used to assess the change in particular pathogenic *Staphylococcus* species levels (Chapter 4). The fold changes in the amount of *S. lentus* bacteria in air samples were calculated. Using SI\_*pfbA*\_FxR primers in qPCR, we observed that the number of *S. lentus* bacteria in the air increases as time goes by in both wire-floored pens A and B during the spring 2021 BCO experiment (Fig. 6.6). During 50–51 d, *S. lentus* was diagnosed in the air samples taken in spring 2021 (Fig. 6.4; Table 6.1).

For the air samples collected during 21–53 d in spring 2022 and the positive control, the average 16S and Saur\_pfbA Ct values were compared (Fig. 6.5). *S. aureus* was found only four times in air samples collected on days 25, 27, and 31 (Table 6.2).

Using the extracted DNA from the air samples collected in the spring of 2021 and 2022 in qPCR experiments, we detected a number of BCO-related pathogens including *S. aureus*, *S. cohnii*, *S. lentus*, *S. haemolyticus*, *Acinetobacter baumannii*, and *Alkanindiges* sp. (Fig. 6.7).

## **Discussion.**

In comparison to typical microbiological culturing techniques to screen for pathogens in agricultural environments, our efficient air sampling system is affordable, quick, repeatable, reproducible, and can be employed in environments with restricted equipment. One of the important characteristics of our technology is rapidity. The sampling takes 5–30 minutes, the DNA extraction takes 30 minutes, and the samples are available for qPCR, which takes about two hours. This air sampling technique can screen for millions of economically important pathogens in the air around economically important animals like chickens, pigs, and cattle without using time-consuming and labor-intensive culture methods (Fig. 6.4).

To monitor airborne infections caused by any bacteria, fungi, protozoa, or DNA viruses that travel via the air, our system should be readily adaptable across a wide range of agricultural and human medical situations, such as hospitals, schools, public transits and places, and the military. The nucleic acid extraction method could be adapted for RNA viruses to other screening needs. This technique is amenable for ensuring food safety and monitoring for agricultural and medically important airborne pathogens.

Future directions automated air samplers with higher flow rates to sample a higher number of airborne pathogens in a shorter time. As a result, by using 16S rDNA and other species-specific primers, one could monitor for pathogens in a number of environments. In addition to species specific probes primers specific to pathogen specific sequences (e.g., mobile elements, pathogenicity islands) could be used to monitor specifically for the pathogenic isolates of particular species, such as *S. aureus* and *Enterococcus cecorum*. This airborne pathogen surveillance system should be adaptable to viruses, fungi, and protozoa that can spread via air.

**Table 6.1.** Air specimens and the methods used to extract DNA from air samples (1–22) collected in the Spring 2021 BCO experiment. Age (day): the birds age during air sampling; Duration (min): The duration of air sampling; CFUs/sample: Viability count of bacteria in every air sample; DNA extraction methods: The methods examined to extract DNA from air samples; Primers: The PCR primers used to target pfbA and/or 16S rDNA genes; qPCR amplif.: qPCR amplification.

Sample#	Age (day)	Duration (min)	CFUs/sample	DNA extraction methods	Primers	qPCR amplif.
1	17	3; 10	4100; 8825	Rapidboiling	(Slen,Sagn,Scoh) pfbA	Yes*
2	20	15; 30	12205; 21050	Rapidboiling	(Slen,Sagn,Scoh) pfbA	No
3	21	20	4500	Mixer_sterile_beads	(Slen,Sagn,Scoh) pfbA; 16S	No
4	27	20	35200	Sonication	Sagn_pfbA; 16S (8Fx936R)	No
5	29	20	4000	Sonication	Sagn_pfbA; 16S; (9_Staph) pfbA	No
6	35	20	17200	Sonication	Sagn_pfbA; 16S (8Fx936R)	No
7	42	20	6000	NaOH+sodium acetate	16S (8Fx936R)	No
8	42	20	6600	Alkaline_lyse	16S (8Fx936R)	Yes
9	44	20	20000	Alkaline_lyse	Sagn_pfbA; 16S (8Fx936R)	Yes
10	44	20	29300	Alkaline_lyse	Sagn_pfbA; 16S (8Fx936R)	Yes
11	46	20	46400	Rapidboiling	16S (8Fx936R)	No
12	46	20	20000	NaOH+C2mimOAc	Sagn_pfbA; 16S (8Fx936R)	Yes
13	48	20	16000	C2mimOAc	16S (8Fx936R)	No
14	48	20	20000	Alkaline_lyse	16S (8Fx936R)	Yes
15	50	20	8000	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
16	50	20	12000	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
17	52	20	9000	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
18	52	20	5000	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
19	54	20	12200	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
20	54	20	12500	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
21	56	20	20000	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
22	56	20	52900	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes
campus**	N/A	20	130	Alkaline_lyse	(Slen,Sagn,Scoh) pfbA; 16S	Yes

\*: We have identified *S. lentus* and *S. cohnii*, but the DNA extraction was not repeatable using Rapidboiling DNA extraction. \*\*: University of Arkansas campus.

Yes: DNA was detected via qPCR amplification. No: DNA was not obtained.

**Table 6.2.** Air specimens and the methods used to directly extract DNA from air samples (1-17) collected during the Spring 2022 BCO experiment, along with the average Ct values obtained from running qPCRs.

Samples	Age (day)	Duration (min)	CFUs/sample	DNA extraction	Primers	Average Ct	
						Saur_pfbA	16S
1	21	20	340	100 mM NaOH	Saur_pfbA; 16S	No	22.3
2	21	20	1520	100 mM NaOH	Saur_pfbA; 16S	No	20.5
3	23	20	1260	100 mM NaOH	Saur_pfbA; 16S	No	19.5
4	23	20	2010	100 mM NaOH	Saur_pfbA; 16S	No	18.3
5	25	20	2080	100 mM NaOH	Saur_pfbA; 16S	35.6*	16.0
6	25	20	3640	100 mM NaOH	Saur_pfbA; 16S	31.3	17.4
7	27	20	4580	100 mM NaOH	Saur_pfbA; 16S	No	20.1
8	27	20	3650	100 mM NaOH	Saur_pfbA; 16S	No	19.3
9	37	20	11200	100 mM NaOH	Saur_pfbA; 16S	No	16.0
10	37	20	12800	100 mM NaOH	Saur_pfbA; 16S	33.0	15.8
11	43	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	15.9
12	43	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	16.6
13	43	20	N/A	100 mM NaOH	Saur_pfbA; 16S	34.1	16.0
14	49	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	25.8
15	49	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	24.3
16	53	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	21.1
17	53	20	N/A	100 mM NaOH	Saur_pfbA; 16S	No	21.1

N/A: CFUs per air sample were not monitored anymore.

\*: We have identified *S. aureus* directly from the air samples, and then sent the PCR product for sequencing to confirm the qPCR result.

No: The *S. aureus* DNA was not detected.

**Table 6.3.** Primers used in the qPCR assays to detect the bacterial 16S and pfbA genes of *Staphylococcus* species collected from air samples in this study.

qPCR Experiment targets	Primer's code	Sequence 5' → 3'	Literature.
Bacterial 16S rDNA gene	8 F	AGAGTTTGATCCTGGCTCAG	Edwards et al., 1989
	936 R	AATTGACGGGGGCCCCGCAC	Baker et al., 2003
<i>S. agnetis</i> pfbA gene	agn_pfbA-F	GCGTAATACGCCAGCACAG TTG	our study
	agn_pfbA-R	CACTTATGGCATTCAACGW GCA	our study
<i>S. aureus</i> pfbA gene	aur_pfbA-F	CTTGAATACCCGCTTCAAYA GGA	our study
	aur_pfbA-R	CRTGCGTTAAATCRTGGAAG ATG	our study
<i>S. capitis</i> pfbA gene	cap_pfbA-F	CSCCAAGAAATCGTATACCA CC	our study
	cap_pfbA-R	GCGAGTAGGGAAAGATGCA TTG	our study
<i>S. chromogenes</i> pfbA gene	chr_pfbA-F	CACTTTGTTGACGTAKTTCC GG	our study
	chr_pfbA-R	GATACGATTGGGATTCAACG YGC	our study
<i>S. cohnii</i> pfbA gene	coh_pfbA-F	GCACTCGAATKGCATCTTTA GAC	our study
	coh_pfbA-R	GGGCAGATACACGYGCGAT TC	our study
<i>S. epidermidis</i> pfbA gene	epi_pfbA-F	CGTAATCGTTCCATCTGTAG TTCC	our study
	epi_pfbA-R	GGCAGACACACTGTTTATAT ACC	our study
<i>S. hyicus</i> pfbA gene	hyi_pfbA-F	TATTAACGTAGCGATGTGGT GC	our study

	hyi_pfbA-R	TACGATTGGAATACAACGG GCA	our study
<i>S. lentus</i> pfbA gene	len_pfbA-F	GTGGAACCAGACTGTGCAC C	our study
	len_pfbA-R	GAAAATCTCCTAGCGCTATG TTG	our study
<i>S. saprophyticus</i> pfbA gene	sap_pfbA-F	CCTGCTTGTGTCCCCCTTTC	our study
	sap_pfbA-R	GGGGAACACAAATGGGCAG	our study



**Table 6.4.** *Staphylococcus aureus* isolates isolated from air samples collected using the air sampling system during days 21, 23, 25, and 27 of the birds' age.

Culture number	Species	Day of air sampling
1738	<i>S. aureus</i>	21
1739	<i>S. aureus</i>	23
1740	<i>S. aureus</i>	25
1741	<i>S. aureus</i>	27

**Table 6.5.** Fold change in *S. lentus* bacterial load in the air samples collected during spring 2021 BCO experiment from both wire-floored pens A and B, using Sl\_pfbA\_FxR primers in qPCR.

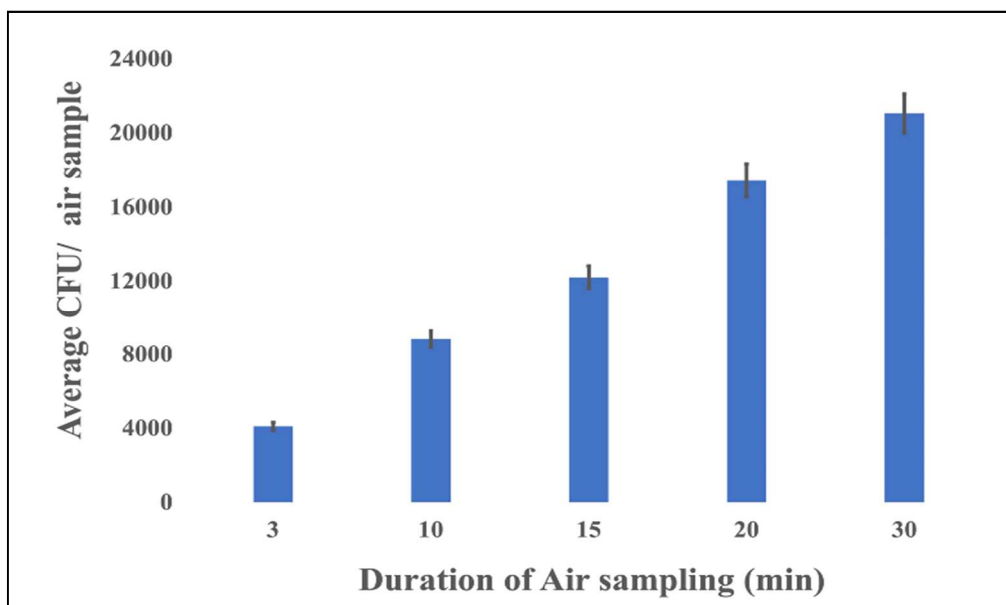
Pen#	A	A	B	B
Day	Fold change CFU	Fold change Sl_pfbA	Fold change CFU	Fold change Sl_pfbA
50	1.0	1.0	1.0	1.0
52	1.1	2.3	0.4	1.1
54	1.5	5.2	1.0	3.3
56	2.5	10.3	4.4	5.4



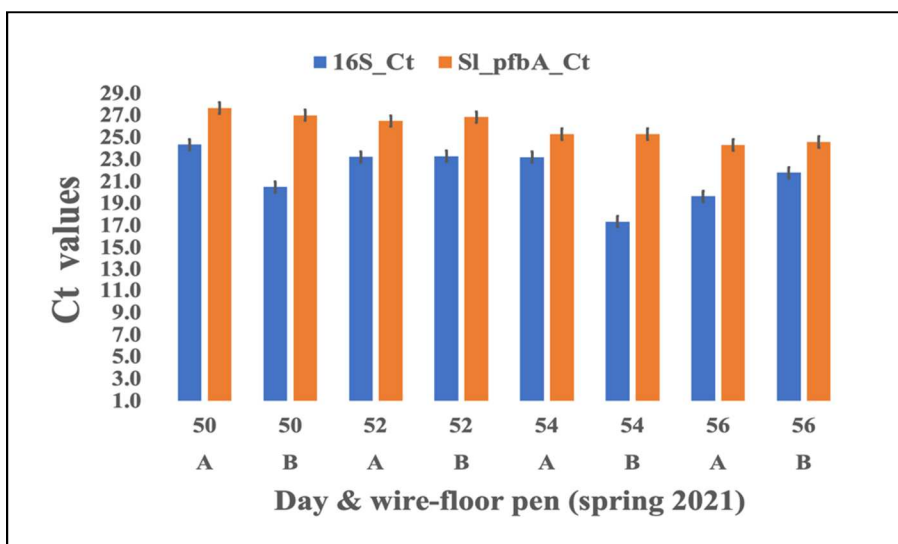
**Figure 6.1.** Colony colors of *Staphylococcus* species isolated from BCO lesions plated on 2 CHROMagar media.



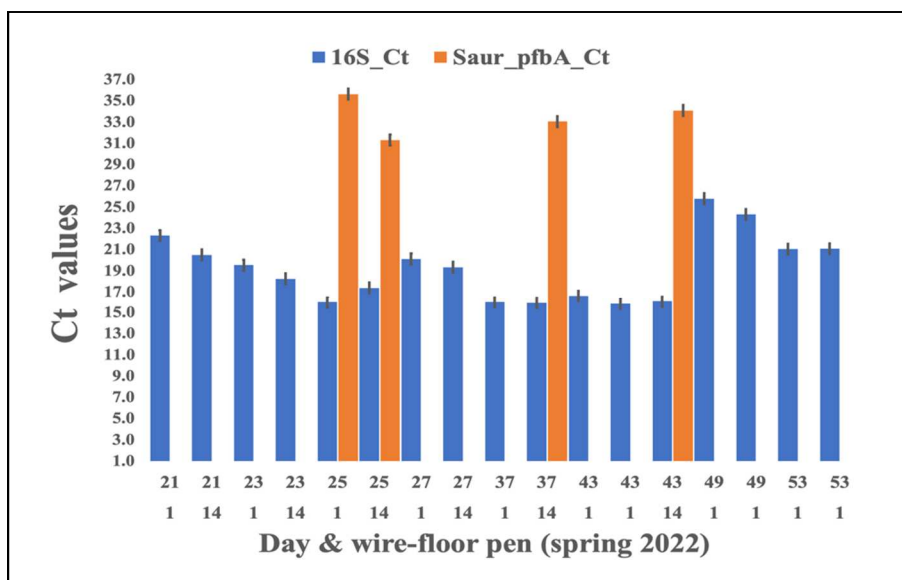
**Figure 6.2.** The air sampling system. composed of an impinger and stopper attached to a portable pipet–pump (e.g., Integra Biosciences Pipetboy acu 2 pipet pump) connected through a 10 cm long 4-mm diameter hose connected to a 5 cm long 5-ml glass pipet piece.



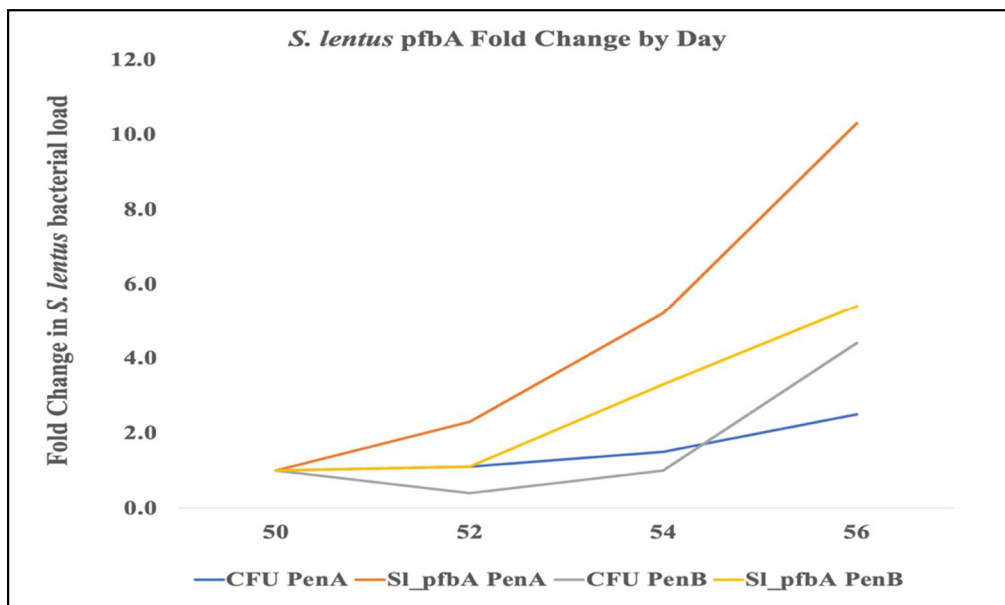
**Figure 6.3.** Average CFU per air sample collected using impinger\_pipet pump system versus different durations of air sampling (minutes).



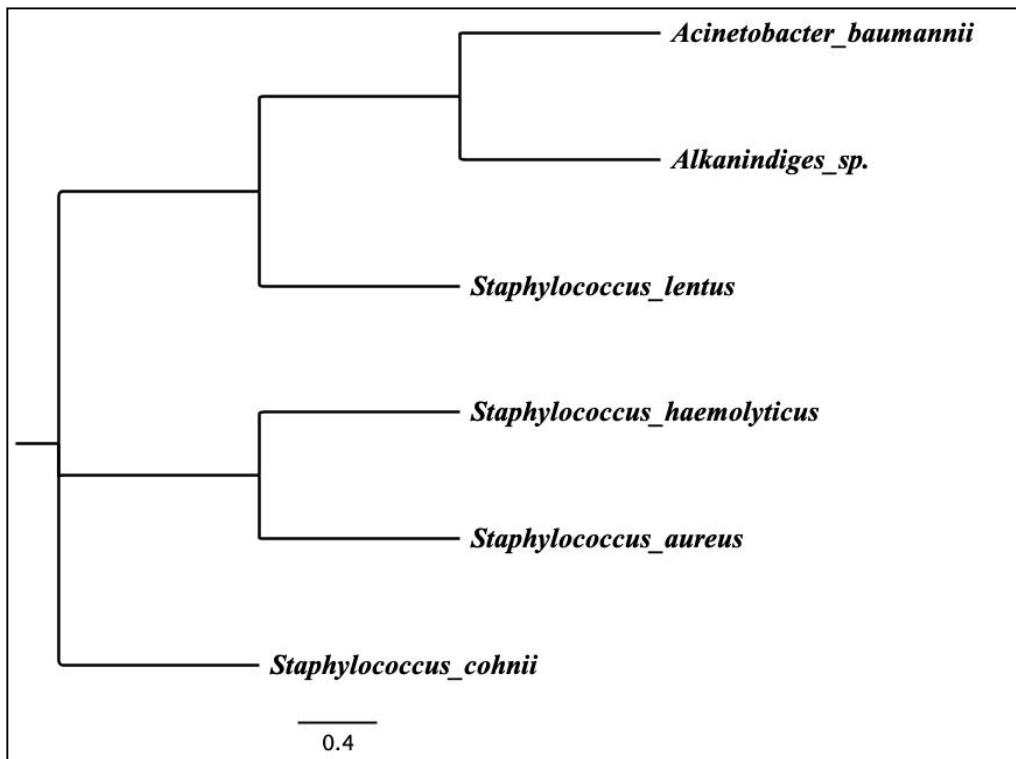
**Figure 6.4.** Average Ct values using bacterial 16S (16S\_Ct) and *S. lentus* pfbA (SI\_pfbA\_Ct) primers for DNAs extracted from air samples collected on days 50, 52, 54 and 56, around the wire floor pen A and B in spring 2021 using our air sampling system.



**Figure 6.5.** Average Ct values using bacterial 16S (16S\_Ct) and *S. aureus* pfbA (Saur\_pfbA\_Ct) primers for DNAs directly extracted from air samples collected on days 21, 23, 25, 27, 37, 43, 49 and 53, around the wire floor pen A and B in spring 2022 using our air sampling system.



**Figure 6.6.** Fold change in *S. lentus* bacterial load and CFUs counted in air samples from both wire-floored pens (A & B) during the spring 2021 BCO experiment, using SI\_pfbA\_FxR primers in qPCR.



**Figure 6.7.** Neighbor Joining tree based on 16S rDNA comparisons using the MegAlign Pro/DNA Star. This tree is built using the 16S rDNA (8Fx936R primers) of the bacteria cultured from air on our farm.

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## **CHAPTER 7**

### **Conclusions.**

## Chapter 7: Conclusions.

The rapidly increasing human population of the globe will lead to an increase in the need for food. Over the last few decades, meat consumption has increased worldwide in parallel with the expansion of the global human population. Poultry meat is an inexpensive source of protein, minerals, and vitamins. Long-term poultry production sustainability while planning for unforeseen scenarios such as COVID-19 is a major issue for the poultry industry. The poultry industry has evolved from smaller home farming to an advanced and sophisticated poultry production system. The industry has successfully selected the traits that are economically significant, including as high meat yields and egg production. One of the consequences of intense genetic selection is lameness. It costs the poultry industry millions of dollars annually with its global prevalence. The leading cause of lameness in commercial broilers is bacterial chondronecrosis with osteomyelitis (BCO). The complete pathogenesis of the disease is not fully known at this time. Nevertheless, we hypothesize that microorganisms are conveyed by vertical and horizontal transmission to birds that acquire lameness via impaired intestine, and respiratory tract. These bacteria are able to survive and colonize long bones as well as vertebrates, resulting in several described types of necrosis. In our study on lameness, *Staphylococcus agnetis* was identified as the predominant agent responsible for BCO-induced lameness in birds. This agent may account for the half of all occurrences of lameness in our farm. Other opportunistic bacteria, like *S. aureus*, and *Escherichia coli*, are associated with the etiology of BCO in broiler chickens in other farms.

In this dissertation, we compared the whole genomes of different *S. agnetis* isolates that infect chickens and cattle. We have also covered phylogenomics and methods for identifying specific diseases during outbreaks. We found a long catalogue of 40 genes and three plasmids

were found in the chicken isolate of *S. agnetis*, while these genes and plasmids were not found absent or poorly conserved in the cattle *S. agentis* isolates.

We continued our search for the virulence genes of the chicken strain of *S. agnetis* that were not present in the bovine isolates. In order to transfer DNA from chicken isolate to bovine isolates, we have refined a dependable electroporation process. Using a Gram+/Gram- shuttle vector, we typically produce 10 to 20 transformants per ng DNA. In the direct genome evolution experiment research, we successfully used the improved DNA transformation methodology.

Among the BCO pathogens that were isolated from the lame broilers, there were a number of *Staphylococcus* species. These included *S. agnetis*, *S. hyicus*, *S. chromogenes*, *S. aureus*, *S. cohnii*, *S. saprophyticus*, *S. epidermidis*, and *S. capitis*. We evaluated several housekeeping and virulence genes in an effort to identify a reliable genetic marker capable of distinguishing *Staphylococcus* species with ease. This investigation identified a significant and reliable gene, plasmin and fibronectin-binding protein A (pfbA). This is the first report on using pfbA for the identification and diagnosis of *Staphylococcus* species.

The process of extracting bacterial DNA for use in later molecular diagnostic applications continues to be time-consuming, labour-intensive, and expensive. We came up with a way to rapidly extract genomic DNA from environmental materials by lysing the cells with sodium hydroxide and either capturing the DNA with or without paramagnetic bead capture. In this study, we explored the effect that varying doses of NaOH had on subsequent quantitative PCR. The DNA of both gram-negative and gram-positive bacteria was successfully extracted using NaOH. We used the optimal concentration of NaOH on a variety of samples derived from the environment, agriculture, food production, and clinical specimens. These samples included air,

soil, sewage, food, laboratory surfaces, and chicken cloacal swabs. Bacterial DNA could be assessed by qPCR by dilution of the bacterial NaOH lysate or the DNA captured from the NaOH solution by magnetic beads. In this study, we examined two different types of paramagnetic beads and used a described efficient buffer for bead binding. The optimized technique of DNA extraction by NaOH is inexpensive, and it can be performed in ten minutes at room temperature. This method is well suited for high-throughput investigations and makes it possible to extract bacterial DNA even in environments with limited resources.

One of the transmission routes of BCO pathogens from is via air. In this work, an effective methodology for collecting samples of airborne bacterial pathogens is presented. Air sample collection utilized an impinger and portable vacuum pump to collect microorganisms from the air within poultry barns. Our air sampling system was designed for the quick screening of these airborne BCO pathogens, and transferable to monitor agriculturally important pathogenic bacteria. A single sample contains enough cells to test for several species, allowing for the detection of a vast array of harmful bacteria from each air sample. Air samples included *S. lentus*, *S. cohnii*, and *S. aureus*, which were recognized by qPCR. In air samples obtained from naturally affected flocks, *S. aureus* and three other BCO-related pathogens were found. Our DNA sample method is far faster, easier, and less costly than conventional culture-based methods. Using the air sampler, we were able to capture  $13,062 \pm 7,600$  CFUs in 20 minutes. The manual operation of the sampler ensures its suitability for detecting airborne infections in situations with limited equipment. The technology is anticipated to be readily transferable to many agricultural systems and even human medical systems in order to screen for airborne infections.