ABSTRACT

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                                            ANTIMICROBIAL RESISTANT
                                            STAPHYLOCOCCUS AUREUS IN RETAIL
                                            GROUND MEATS

                                            Yi Li, Masters of Science, 2010

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Staphylococcus aureus is commonly present in humans and animals. It can cause a
variety of suppurative infections, food intoxication and toxic shock syndrome.
Antimicrobial resistant *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA),
have emerged and are a major public health concern. There is an increasing risk of
food production animals serving as a reservoir and transmitting *S. aureus* and MRSA
in community environments. Due to the increased food safety risk posed by MRSA in
addition to its multidrug resistance, we were interested in determining the prevalence
of *S. aureus* and MRSA in retail meat and investing the multidrug resistance of the *S.
aureus* isolates. A survey study was conducted, involving 480 retail ground meat
samples (231 ground pork and 249 ground beef) collected in the Washington DC area
from March 2009 to March 2010. Approximately 42.08% (n = 202) of the samples
were identified as *S. aureus* positive and one MRSA isolate was recovered from a
ground beef sample. Antimicrobial resistance testing showed 53.34% of recovered *S.*

aureus isolates exhibited different levels of antimicrobial resistance to CLI, CHL, GEN, LEVO, CIP, SYN and TGC. The MRSA isolate was resistant to 8 of 22 antimicrobials tested. PFGE fingerprinting identified the MRSA isolate as USA300 subtype, which also carried genes of virulence factors PVL and protein A. Our findings indicated that antimicrobial resistant *S. aureus* strains were common in retail ground beef and port, and that MRSA could also be present in such products that could potentially serve as a reservoir.

ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANT
STAPHYLOCOCCUS AUREUS IN RETAIL GROUND MEATS

By

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Table of Contents

List of Tables

List of Figures

Chapter 1: INTRODUCTION

Microbiology of *Staphylococcus aureus*

In 1880, *Staphylococcus aureus* was first discovered by a surgeon named Sir Clifton Smith in pus from surgical abscesses in Aberdeen, Scotland (1). It is a non-motile, non-sporeforming, Gram positive, facultative anaerobic coccus, with the appearance of grape-like clusters when viewed through a microscope. The *S. aureus* spherical cells are about 1 micrometer in diameter, and form a cluster shape because of the special division way of *Staphylococci*, the cells dividing in both three dimensional axis and the new cells remaining attached to each other followed by each division successively. Since there is no exact point of division, the result is to form an irregular cells cluster (2). *S. aureus* is catalase positive and oxidase negative. When without a microscope, the catalase test is an important but very simple method to distinguish *Staphylococci* from *Streptococci*, which is catalase negative (3). Typical *S. aureus* has a large, round, creamy smooth colonies with golden yellow color. Most strains have beta or alpha hemolysis when growing on blood agar plates (4). *S. aureus* can survive for several hours on dry environmental surfaces (5), and grow at a temperature range of 7 to 48°C. It can tolerate NaCl concentrations as high as 15 percent (6). From the sequenced *S. aureus* strains, the range of genomes size is around 2.8 to 2.9 Mbp (7-9), which include 75% essential genes for cell surviving and other accessory genome, bacteriaphages and pathogenicity island that contains various virulence genes (Table 1-1) (7, 9-10).

Table 1-1. *Staphylococcus aureus* genomics

Genomic Characteristics	
Size of genomic DNA (Mbp)	2.8 - 2.9
Predicted protein-coding sequences (genes)	2565 - 2671
Percentage of core component genes (%)	75%

There are more than 20 species of *Staphylococcus* described in Bergey's Manual (2001) (11), but only *S. aureus* and *Staphylococcus epidermidis* have significant impact on human health. When growing on a nutrient media, brain heart infusion (BHI) agar for instance, *S. aureus* normally produces bigger, yellow, creamy colony, and hemolysis should be observed when grown on blood agar plates. *S. epidermidis* has a smaller white colony with no hemolysis. In addition, nearly all *S. aureus* are coagulase positive and most *S. epidermidis* are coagulase negative (12-13). Unlike *S. aureus*, which should always be considered as pathogen, the majority of *S. epidermidis* is nonphathogenic and belongs to a part of the normal flora that plays a role of protection. However, *S. epidermidis* may be considered as a pathogen in a clinical environment (14).

Reservoir of *S. aureus*

S. aureus can survive in various environments and has a wide range of natural reservoir (15). It may occur as a commensal flora on human skin and also in the nose and most other anatomical locales, including oral cavity and gastrointestinal tract. Approximately 20% of the human populations are long term *S. aureus* carriers and about 60% of the population can be colonized by *S. aureus* intermittently. Intravenous drug users, patients with history of long term healthcare, immunocompromised

person and healthcare workers tend to have a higher rate of *S. aureus* carriage (15-16). However, the presence of *S. aureus* under those circumstances does not always indicate an infection occurred. In addition, *S. aureus* can also be present in many domesticated animals such as cats, dogs or horses (18-20). Food production animals are another important reservoir for *S. aureus*. Pigs and cows are generally colonized by *S. aureus* at farming condition. It causes septicemia in pigs and responsible for majority of mastitis cases in cattle (21-22).

Virulence factors of *S. aureus*

    The majority of *S. aureus* strains can express more than one virulence factor. Various virulence or virulence-like genes on *S. aureus* chromosome code for surface virulent structures and secreted proteins as well as other regulators which help it adapt to the host environments. (Figure 1-1)
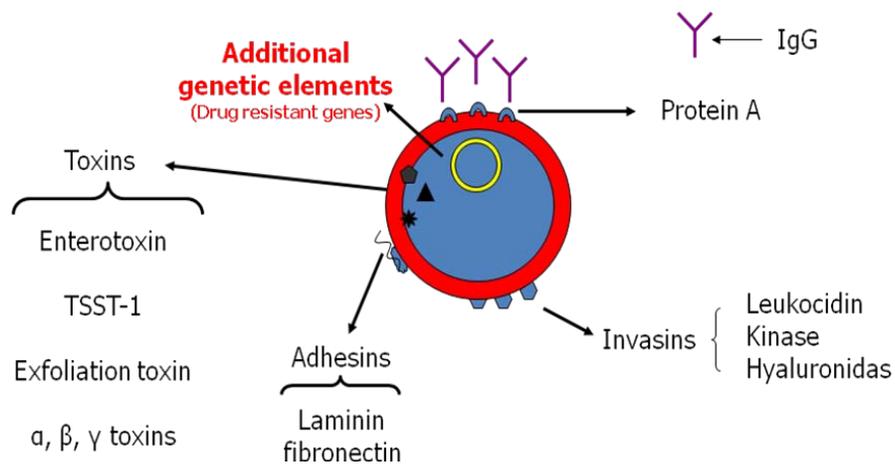
Figure 1-1. Virulence factors of *S. aureus* Figure 1-1. Virulence factors of *S. aureus*

3

Among various potential virulence factors, seven groups have been identified as the major causes of *S. aureus* infections:

1. Adhesins

   Adhesins are surface proteins that promote the colonization of host tissues (23). *S. aureus* cells can express several surface proteins to help the bacteria attach to the host cells. Laminin and fibronectin are two major components that form an extracellular matrix on the epithelial or endothelial surfaces. In addition, the expression of fibrin or fibrinogen binding protein is very common in *S. aureus*. These are clumping factors that can help the bacterial cells to attach to blood clots and traumatized tissue. Most *S. aureus* strains can express both adherence factors (23-25).

2. Invasins

   Invasins of *S. aureus* are a group of extracellular proteins that promote bacteria to spread into tissues, such as leukocidin, kinases, and hyaluronidase (26). Leukocidin is one of the primary virulence factors in *S. aureus*, and is a multi-component protein, which has been produced separately and acts together as a membrane pore forming toxin. The target of this virulence factor is polymorphonuclear leukocytes (27).

3. Surface factors

   S. aureus can produce various surface factors that help it inhibit the phagocytic engulfment. Amount these surface factors, protein A is the most important virulence determinant. It is a surface protein that can bind to the host IgG

molecular at a wrong orientation, which leads to the disfunction of IgG protein and eventually disrupts phagocytosis and opsonization (23, 28).

4. Superantigens (PTSAgs)

S. aureus secretes two different toxins that have superantigen activity. The secreted superantigens stimulate the non-specific activity of T cell without a normal antigenic recognition. Cytokines are released in a large amount and lead to disease symptoms (26). One is enterotoxin which has six subtypes: SE-A, SE-B, SE-C, SE-D, SE-E and SE-G. Enterotoxins can cause food poisoning by cause GI tract symptoms, such as diarrhea and vomiting, after they are ingested. Another superantigen that *S. aureus* commonly produced is toxic shock syndronme toxin (TSST-1), which is the cause of toxic shock syndrome (TSS) (29).

5. Exfoliatin toxin (ET)

Exfoliatin toxin has two distinct antigenic forms, ETA and ETB. They both have protease activity that causes separation within the epidermis, through the stratum granulosum layer of the epidermis, between the superficial dead layers and the living layers. ET is related with the staphylococcal scalded skin syndrome (SSSS), which commonly occurs in infant or young children (29).

6. Other toxins

S. aureus also express a variety of other membrane damaging toxins including alpha-toxin, beta-toxin, delta-toxin and several bicomponent toxins. Alpha-toxin is a pore forming toxin, which also called as alpha-hemolysin. Human platelets and monocytes are especially sensitive to the toxin (30). Beta-toxin targets

sphingomyelin and damages membranes with rich lipid (31-32). The rules of delta-toxin remain unknown. It is a very small peptide that can be expressed by most *S. aureus* strains and some *S. epidermidis*.

7. Antimicrobial resistance elements

S. aureus can also acquire antimicrobial resistance through plasmid and other mobile elements. More than one antimicrobial resistance gene can be located at *S. aureus* simultaneously and leads to multidrug resistance (33). In addition, efflux pump systems also work as antimicrobial resistance elements in *S. aureus* (34-35).

Antimicrobial Resistance of *S. aureus* and MRSA

Before the first antimicrobial was discovered, the mortality of *S. aureus* bacteremia was over 80%, and more than 70% of patients developed metastatic infections (36). In early 1940s, the introduction of penicillin significantly increased the survival rate of *S. aureus* infections. However, only 2 years later, the first penicillin resistant *S. aureus* was isolated in a clinical environment, and since then resistance pattern has been spreading from hospital to community (15). Penicillin is inactivated by penicillinase (beta-lactmase) (36-37).

New antimicrobials have been continuously introduced. However, widespread and inappropriate use of antimicrobials, along with lacking of efficient antimicrobial administration, large scale use of antimicrobial agents in animal feed as growth enhancers and increased international population movements, has contributed to the increasing of antimicrobial resistance in many microbial pathogens including *S.*

aureus (38-39). As rapidly as a new antimicrobial is introduced into commercial use, *S. aureus* develops efficient mechanisms to neutralize it.

Methicillin, as the first beta-lactamase resistant penicillin, was used to treat *S. aureus* infection in 1961 (40). The first methicillin-resistant *S. aureus* (MRSA) was identified in the United Kingdom in the same year. It appeared in the United States in 1981 among intravenous drug users (43). Although there is no exact definition for MRSA currently, MRSA is commonly referred to as multidrug-resistant *S. aureus* or oxacillin-resistant S. *aureus* (ORSA). It is a special group of *S. aureus* that have acquired the ability to resist a large group of antimicrobials called the beta-lactams, including penicillin, methicillin, dicloxacillin, oxacillin and cefoxitin. In the United States, there is a significantly increase number of MRSA infections during the past decade. A 2007 report from Centers for Disease Control and Prevention (CDC) showed that the number of MRSA infections in clinical environment was doubled nationwide. The annual number of the infected cases increased from approximately 127,000 cases in 1999 to 278,000 in 2005, and the annual death number also increased from 11,000 to more than 17,000 at the same time period (44). When comparing with patients without normal *S. aureus* infections, a 2004 study indicated that in the United States the patients who suffered from MRSA infections had approximately three times the length of hospitalization and three times the total cost. In addition, the risk of death increases by five times (45). Several other studies also show that MRSA contributed to a higher mortality of infection than methicillin-susceptible *S. aureus* (MSSA) (46). Currently, MRSA is divided into two subgroups: the healthcare associated MRSA (HA-MRSA) and community associated MRSA

(CA-MRSA). HA-MRSA is the major problem in nosocomial infections. For instance, patients in hospital with open wounds, invasive devices or under immunocompromise conditions are at much higher risk of getting MRSA infection. On the other hand, the CA-MRSA has recently risen as a major public health concern. Although the border between HA-MRSA and CA-MRSA are not clearly distinguishable, CA-MRSA infections are generally caused by the MRSA strains that differ from the HA-MRSA strains. Comparing with the better understood HA-MRSA, CA-MRSA has its own new characteristics that are still under study (47). Also, CA-MRSA can be defined by characteristics of patients who develop MRSA infections including no contact of healthcare facilities and no history of longtime hospitalization. In the mid-1990s, the first CA-MRSA case was reported in Australia. Soon after that, CA-MRSA infections have been reported in many countries including France, Finland, New Zealand and the United Kingdom. In North American, the United States and Canada also have had reported cases (48-50). In United States, the new CA-MRSA strains have rapidly become the most common cause of skin infections among individuals out of hospital environment. Unlike HA-MRSA, these strains tend to infect much younger populations. The average age of infection population is under 20 years old (51). To date, several reports have documented CA-MRSA infections due to the contact of food animal or consumption of food products (52-57). Several studies have reported MRSA strains from various food products including meats, dairy products and even other processed food including salad dressing or sandwich fillings (92, 93, 103, 104).

At the molecular genetics level, when comparing with traditional *S. aureus* strains, MRSA strains contain additional motile chromosomal DNA fragment, which is called *Staphylococcus* Chromosome Cassette *mec* or *SCCmec* genomic island. The genetic element is approximately 30 to 50 kb (33, 58-59). On the *SCCmec*, it contains *mec*A gene encoding penicillin binding protein (PBP) 2a or 2', and regulatory genes *mec*I and *mec*R1 that are located immediately at upstream of the *mec*A promoter (58-59). Studies reported that the original *mec*A gene may come from a coagulase-negative *Staphylococcus* species, most possibly *S. sciuri*, or a close evolutionary relative of this species (60). The PBP-2a protein encoded by *mec*A is an inducible 76 kDa PBP responsible for the methicillin resistance (60).

In general, *S. aureus* produces four major PBPs, PBP 1 to 4, which catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. It is essential for the cell wall synthesis, but beta lactam antimicrobials can bind to the original PBP active site and thereby inhibit the cell wall synthesis. However, PBP-2a, which has a reduced affinity for binding with beta lactam antimicrobials (61-62), can substitute for the essential cell well synthesis function of original high-affinity PBPs at the lethal concentration of antimicrobials therefore the bacteria survive.

In additional to resistance to methicillin, MRSA strains commonly exhibit multidrug resistance. Because of the higher antimicrobial pressure in clinical environment, HA-MRSA tends to have a higher antimicrobial resistance spectrum than CA-MRSA. In addition to *mec*A gene, *SCCmec* cassette may contain other antimicrobial resistance genes. Both *S. aureus* and MRSA can harbor various

resistance genes on chromosome and/or plasmids. For instance, *van*A gene, which is responsible for vancomycin resistance, is located on a 60 kb plasmid (63). On another aspect, efflux pumps also contribute to a certain level of antimicrobial resistance in *S. aureus* (34-35).

<u>*S. aureus* pathogenesis</u>

Each year about 500,000 patients in American hospitals contract a *Staphylococcal* infection (17). Human *staphylococcal* infections are frequent. However, in most cases it usually remains localized at the portal of entry by the normal host defenses. The portal of infection usually is a break in the skin including a minute needle-like stick to a surgical wound, most commonly happens for burning wounds (20, 51). Another important portal of entry is the respiratory tract. Pneumonia caused by *S. aureus* is a frequent complication of influenza infection. The localized host response to *Staphylococcal* infection is inflammation which is characterized by an infection site temperature elevation. Swelling, pus accumulation and tissue necrosis may be caused by the immune response of leukocytes and a fibrin clot may form around the inflamed area to wall off the bacteria. In some cases, more serious infections of the skin may also occur, such as furuncles or impetigo. In addition to various superficial infections, severe deep site infections may occur if the bacteria get into organs or deep tissues. *S. aureus* can cause infections in the kidney which may lead to kidney failure, infect the heart causing endocarditis, and invade meninges leading to meningitis (26). Osteomyelitis can occur as the result of the localized bone infection. Septicemia as a serious consequence of *Staphylococcal* infections when the

bacteria invade the blood stream may be fatal and a bacteremia may result in seeding other internal abscesses.

Additionally, various types of toxins produced by *S. aureus* can lead to different diseases. In infants, *S. aureus* infection can cause *Staphylococcal* Scalded Skin Syndrome (SSSS) by ET toxin (26). Some strains of *S. aureus*, which produce exotoxin TSST-1 into the blood stream, are the causative agent of Toxic Shock Syndrome (TSS) (64). Currently, TSS is highly related with tampon use in women, and TSST-1 is responsible for all menstrual cases and also 75% of total cases (65-66).

S. aureus can also cause gastroenteritis by releasing enterotoxins into food products. Symptoms include nausea, vomiting, abdominal cramps and diarrhea (52, 64). In some severe cases, headache, muscular cramps or blood pressure change may also occur. The gastroenteritis is normally self-limited and the symptoms begin release generally in 8-24 hours. However, death may also happen for infants or old people. If the enterotoxin is released systematically in human body, it can also cause TSS. In fact, 50% of non-menstrual cases of TSS are caused by enterotoxins B and C.

MRSA infection has its own unique features. Followed by the initial typical symptoms, MRSA infection progresses substantially within the next 24 to 48 hours. After 72 hours, MRSA can get into deeper human tissues and become resistant to treatment. The initial symptom of MRSA infection show a small red spider bites bumps that may be accompanied by fever and occasionally rashes (67-68). Within a few days the spider bites bumps become larger, more painful and eventually open into deep, pus-filled boils (68-69). MRSA can be very hard to eliminate because of its ability to resist to one or even more antimicrobial agents. The MRSA incubation

period for healthy individuals varies from a few weeks to many years asymptomatically (70). Patients with compromised immune systems are at a significantly higher risk of symptomatic secondary infection (45). Compare with HA-MRSA, CA-MRSA display some characteristics, such as enhanced virulence, more rapidly spreading, which lead to a higher risk to get more severe diseases (71-73).

For the majority of diseases caused by *S. aureus*, pathogenesis is multi factorial. It is difficult to precisely determine the role of any given virulence factor (74). However, evidences have been found that the expression of particular virulence determinants have correlations with strains isolated from particular diseases, which may suggests their specific role in particular diseases. In addition, the application of various molecular biology methods has led to advances in unraveling the pathogenesis of *Staphylococcal* diseases. Genes which encoding potential virulence factors have been sequenced and cloned, various toxin proteins have been identified and purified. With some *staphylococcal* toxins, symptoms of human disease can be reproduced in animal models with the purified protein toxins, lending to better understanding of mechanism of action (7, 75-77).

Laboratory Diagnosis

The diagnosis of *S. aureus* infection depends upon the symptoms of patient and the healthcare advisor's evaluation. Obtaining an appropriate specimen is the first step of definitive diagnosis of *S. aureus* infection. Based on the type of infection presented, the specimen can be collected accordingly and sent to a laboratory for identification by biochemical, enzyme-based or molecular-based tests.

Under laboratory condition, a Gram stain can be performed as the first identification step. If Gram positive cocci are observed under microscope, further tests should be performed. Secondly, culturing the suspect specimen on selective medium, such as Mannitol Salt Agar (MSA), BP agar or other chromogenic agars to differentiate *Staphylococci* or *S. aureus* from other bacteria, can be used to obtain the typical single colony. Typical *S. aureus* should show a big creamy yellow colony surrounded by yellow area on MSA or Big glossy black colony surrounded by clear hole on BP agar. Under certain cases, selective medium that contain specific antimicrobials may used to identify the antimicrobial resistant ability of the isolates. For instance, BP agar with 4µg/ml oxacillin can be used to identify MRSA from specimen. MIC test is also needed to determine the antimicrobial susceptibility.

To differentiate on the species level, several biochemical tests can be performed, such as catalase test and coagulase test. A typical *S. aureus* isolate show a strange catalase positive and coagulase positive results. For the confirmation of diagnosis or subtyping of the obtained isolates, molecular based methods are currently the most popular choose. Among all of molecular methods, PCR for *nuc* or *fem* gene is the most commonly used to confirm isolate identification as *S. aureus*.

Pulsed-field gel electrophoresis (PFGE) can be used to sub-categorize MRSA isolates from USA100 to USA1200 (78). In addition, enzyme based assays such as ELISA against protein A are also a good choose for diagnosis. Multilocus sequence typing (MLST) is an effective methods that can be used to detect the different types of *SCCmec* elements among MRSA isolates (79).

For diagnosis, there is no exact "the one" method, especially for MRSA. Based on different situations, various combinations of currently valid diagnostic methods can be determined case by case.

Treatment of *S. aureus* infections

The first choice of treatment for *S. aureus* infection is penicillin. However, since penicillin-resistance is extremely common in many countries, the penicillinase-resistant antimicrobials such as oxacillin or methicillin was developed as the first-line therapy to treat penicillin resistant *S. aureus* infections. Combination therapy with gentamicin may be used to treat severe infections like endocarditis (80), but its use is controversial because of its high risk of kidney damage (81).

Both HA-MRSA and CA-MRSA are resistant to most anti-beta-lactamase antimicrobials. HA-MRSA has a greater spectrum of antimicrobial resistance; it is often susceptible only to vancomcin (82). CA-MRSA may be susceptible to sulfa drugs, tetracyclines and clindamycin, but the drug of choice for treating CA-MRSA has not been established (21). Newer drugs, such as linezolid may be effective against both HA-MRSA and CA-MRSA, but the susceptibility also keeps reducing recent years (83-84). Currently, the fisrt-line treatment for severe invasive MRSA infection is glycopeptides antimicrobials, such as vancomycin. However, this type of antimicrobial has no oral preparation available and its side-effects for human body are also the mainly concern (81). In addition, a particularly concern about using glycopeptides antimicrobials to treat meninges and endocarditis is that these drugs do

not penetrate into the infected tissues very well, and the susceptibility also reduced (85-86).

Because of the existing high level of resistance in MRSA and the increasing resistance to vancomycin, CDC published a guideline for using vancomycin appropriately. And methicillin-sensitive *S. aureus* must not be treated by glycopeptides as outcomes are inferior (87-88). A new antibiotic, platensimycin, was recently identified and has shown promising potential against MRSA (46, 87). Phage therapy can be another potential treatment option (89), and showed its effectiveness against *S. aureus* infection in mice (90). In addition, it has been reported that maggot therapy to clean out necrotic tissue of MRSA infection has been successful (91).

S. aureus and MRSA in food products

Unlike other common foodborne pathogens such as *E. coli* or *Salmonella*, *S. aureus* does not cause illness by the bacteria itself. Instead, the enterotoxin produced by *S. aureus* can lead to food poisoning under certain conditions when the food has been contaminated by *S. aureus* (56, 64). Since *S. aureus* is ubiquitous and very common to be found at domestic animals, food contamination with the pathogen is common (53, 92-93). Various food products are frequently contaminated by *S. aureus* and incriminated for *S. aureus* food poisoning including meat products, eggs, dairy products, vegetables and other processed food such as sandwich fillings or chocolate éclairs (92). Also, inappropriate food handling during preparation may bring *S. aureus* into food and temperature abuse after preparation is commonly involved with food poisoning too. Except the food handlers, equipments and environment surfaces

are also responsible for *S. aureus* contamination. Food poisoning could happen if the contaminated food has not been kept in the right temperature range, below 7.2°C or greater than 60°C (94-95). In addition, contact with the contaminated food products or food handler may also bring the risk of various *S. aureus* infections (96).

Since *S. aureus* can colonize on various sites of food animals asymptomatically, such as pig or cow. These animals may serve as reservoir and/or a transmission vehicle of spreading *S. aureus* and MRSA. Food products derived from the animals may be contaminated with *S. aureus* or MRSA during slaughtering and processing (54). MRSA has been isolated from meat or dairy products in several countries including Netherlands, Italy, Australia, Japan and United States (53, 56, 92, 93). More studies are needed to determine the role of meat products as a potential source of MRSA, and to investigate a possible link of meat contamination to CA-MRSA infections.

Study Objectives

1. To determine the prevalence of *S. aureus* and MRSA in retail meats.

2. To study antimicrobial susceptibility of *S. aureus* and MRSA.

3. To characterize MRSA isolates using molecular typing techniques.

Chapter 2: MATERIALS AND METHODS

<u>Sampling</u>

To assess the prevalence of *S. aureus* and MRSA in retail meats, 480 meat samples (231 ground pork and 249 ground beef) were collected weekly from three local grocery stores of three major chains in the Washington D.C. area from March 2009 to March 2010. Each week 12 samples including 2 ground beef and 2 ground pork were randomly collected from each store.

<u>Isolation of *S. aureus* and MRSA from retail meats</u>

Each meat sample (50 g) was added to a plastic filter bag (Fisher, Pittsburg, PA) with 100 ml buffered peptone water (BPW, BD Diagnostic Systems, Sparks, MD) and followed by mixing at 230rpm for 1 minute in a stomacher (Seward, Bohemia, NY). Then 3 ml of the meat rinse were added to 27 ml of modified tryptic soy broth (mTSB, BD Diagnostic Systems, Sparks, MD) which contained 6.5% NaCl and 1% sodium pyrate (Fisher, Pittsburg, PA), and incubated at 35℃ for 48 hours with shaking at 150rpm. Bacterial genomic DNA was extracted directly from the enrichment broth and a PCR pre-screening was performed. Enrichment broth (1 ml) from each sample was added to a 1.5 ml centrifuge tube, followed by centrifugation at 13,000 rpm for 30 s. After removing supernatant, a second centrifugation at 13,000 rpm for 30 s was applied. The remaining supernatant was removed, and bacterial cells were resuspended with 500 μl DD water and vortex for 1. Bacterial DNA was

17

exposed by boiling the bacterial mixture at 100℃ heat block for 15 min. After

heating, the tube was centrifuged at 12,000xg for 5 min. The supernatant was used

for DNA template in a multiplex PCR targeting *S. aureus* specific gene *nuc* and

methicillin resistance gene *mec*A (Table 2-1). The PCR was performed at a 25 μl

reaction solution containing 2 μl of DNA template, 2.5 μl of 10×PCR buffer, 2 μl of a

1.25 mM mixture of deoxynucleoside triphophate, 4 μl of 25 mM $MgCl_2$, and 0.25 μl

of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ)

and 0.25 μl (12pmol) *mec*A and 0.25 μl (6pmol) *nuc* oligonucleotide primer

(Invitrogen, Carlsbad, CA). Thermocycling protocol included an initial denaturation

at 94 °C for 10 min, followed by 30 cycles of denaturation (94 °C for 15 s), annealing

(55 °C for 30 s), and extension (72 °C for 30 s), and a final extension at 72 °C for 10

min. PCR product (4 μl) was mixed with 1 μl loading buffer and added to a 2%

(wt/vol) agarose gel, and an electrophoresis was run at 100 mV for 40 min. Gel was

stained with ethidium bromide and the DNA bands were visualized and photographed

under UV illumination at BIO-RAD Universal Hood II imaging system (Bio Rad,

Hercules, CA). MRSA strain ATCC43300 containing both *nuc* and *mec*A genes was

used as the positive control. After the multiplex PCR pre-screening, a broth sample

with *nuc* and/or *mec*A positive were spread on Baird Parker (BP) agar (BD

Diagnostic Systems, Sparks, MD). In addition, if a broth sample showed positive of

the two target genes, it was also spread on a BP agar with 2μg/ml cefoxitin. After

being incubated at 35℃ for 48 hours, 2-4 typical *S. aureus* colonies from BP agar and

all typical *S. aureus* colonies from BP agar + 2μg/ml cefoxtin were streaked on Blood

Plate Agar (BPA) and incubated at 35℃ for 24 hours. Colonies with big, yellow and

creamy appearance on BPA were saved in BHI broth with 50% glycerol at -80°C for further study.

Colony Hybridization

In order to increase the probability of isolating MRSA, colony hybridization that directly targeted *mec*A gene was performed for broth samples positive of both *nuc* and *mec*A genes at PCR pre-screening. A DNA probe specific for *mec*A gene was generated by PCR. The PCR reaction contained 2 µl of DNA template, 2.5 µl of 10×PCR buffer, 2 µl of a 1.25 mM mixture of labeled deoxynucleoside triphosphate (dNTP+ddNTP) (Roche, CH-4070 Basel, Switzerland), 4 µl of 25 mM $MgCl_2$, and 0.25 µl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 0.5 µl (25pmol) of each oligonucleotide primer (Invitrogen, Carlsbad, CA). Thermocycling protocol included an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 15 s), and extension (72 °C for 45 s), and a final extension at 72 °C for 10 min. Enrichment broth samples positive of *nuc* and *mec*A by PCR were diluted 500,000 to 1,000,000 times using Buffer Peptone Water. 200 µl diluted broth was spread on a TSA agar (BD Diagnostic Systems, Sparks, MD) and incubated at 35℃ for 24 h. The TSA plates with approximately 100 to 200 colonies were chosen for hybridization template plates. GE positive charged Nylon membrane (GE, Minnetonka, MN) was cut to fit the template plate and then marked for orientation, and placed on the surface of a prepared template plate for about 5 min. After the colonies were copied to the Nylon membrane, the template plate was stored at 4°C for the isolation of positive

colonies. The Nylon membrane was lysed in the denature solution (0.5 M NaOH, 1.5 M NaCl) for 15 min with bacteria side up, and placed in neutralization buffer (1.5 M NaCl, 1 M Tris-HCl, PH 7.4) for 15 min, followed by using 2 X SSC (0.3 M NaCl, 0.03 Sodium citrate) washing for 10 min and then dried on a dry 3 MM papers for 1 min. GS GENE UV crosslinker (Bio Rad, Hercules, CA) was used to crosslink bacteria DNA to the Nylon membrane for 5min under C-L energy program. After the DNA was fixed, the membrane was transferred into 200 ml 6 X SSC solution (prewashing solution) and incubated in water bath at 50°C for 30 min with 150 rpm shaking. Cell debris on the surfaces of the membrane was gently removed by using Kimwipes soaked with warm prewashing buffer. A pre-hybridization step was performed in a hybridization tube (Fisher, Pittsburg, PA) with 15ml DIG-Easy-Hyb buffer (Roche, CH-4070 Basel, Switzerland) at 42°C for 1 h, and hybridization was carried out at 42°C overnight with 6 ml DIG-Easy-Hyb buffer containing 12 μl labeled *mec*A probe. After hybridization, the membrane was carefully pulled out from the hybridization tube, followed by washing twice in 2 X SSC solutions with 0.1% SDS (low stringency washing) at room temperature for 5 min. The washing step was repeated twice under condition of 0.1 X SSC solusion with 0.1% SDS (high stringency washing) at 68°C for 15 min. The presence of labeled probes was detected by alkaline phosphatase-conjugated antibody detection kit (Roche, CH-4070 Basel, Switzerland) and NBT/BCIP stock solution (Roche, CH-4070 Basel, Switzerland). The membrane was briefly rinsed in washing buffer for a few seconds and then incubated at room temperature for 30 min in 100ml blocking solution, and incubated for another 30 min in 20 ml freshly prepared antibody solution, followed by 2 times

15 min washing with 100 ml washing buffer. After washing, the membrane was equilibrated in 20 ml detection buffer for 2-5 min, and then transferred into 10 ml detection buffer with 800 μl NBT/BCIP stock solution for the coloration reaction in an appropriate container with dark environment and no shaking. After about 3-12 hours, the membrane was washed with 50 ml of sterile double distilled water to stop the reaction when desired purple spots intensified were achieved. Then the membrane was compared with the original template plate with the marked orientation, and up to 15 original colonies on the template plate were picked out to BPA and grown at 35°C for 24h. The positive isolates were then saved in BHI broth with 50% glycerol at -80°C for further study.

S. aureus and MRSA confirmation

All collected isolates were confirmed by PCR and other biochemical tests for identification. The multiplex PCR target *nuc* and *mec*A genes was followed the same PCR experimental details as the PCR pre-screening. In addition, for each *S. aureus* isolate, the hemolysis morphology was observed on the BPA agar. A few colonies from BPA was streaked out and added to a 1.5ml centrifuge tube with 0.5ml coagulase rabbit plasma w/EDTA (Remel, Lenexa, KS) for coagulase test. After 2 hours incubation at 35℃, the result was recorded based on the agglomeration in the tube. If the reaction was negative after 2 hours, the tubes were remained at 35℃ up to 18 hours. In addition, one colony from BPA was streaked on a clean glass slide and added with one drop of hydrogen peroxide solution for catalase test (generating

bubbles indicate a positive reaction). The combined information from all the tests was used to evaluate if the isolates were confirmed as *S. aureus* or MRSA.

Antimicrobial susceptibility test

In order to characterize the antimicrobial resistant profile of *S. aureus* and MRSA isolates that recovered from retail meats, the Sensititre MIC susceptibility system (TREK Diagnostic Systems, Cleveland, Ohio) was used to simultaneously investigate the susceptibility to 22 different antimicrobials for saved *S. aureus* isolates. Minimum inhibitory concentrations (MIC) of 19 antimicrobials were determined including Chloramphenicol (CHL), Erythromycin (ERY), Clindamycin (CLI), Daptomycin (DAP), Oxacillin+2%NaCl (OXA), Gentamicin (GEN), Ampicillin (AMP), Linezolid (LZD), Penicillin (PEN), Rifampin (RIF), Vancomycin (VAN), Trimethoprim/Sulfamethoxazole (SXT), Levofloxacin (LEVO), Ciprofloxacin (CIP), Quinupristin/dalfopristin (SYN), Tigecycline (TGC), Nitrofurantoin (NIT), Tetracycline (TET) and Moxifloxacin (MXF). Four additional antimicrobials resistances were also tested including high level streptomycin resistance (STR 1000 µg/ml), inducible Clindamycin resistance (DT 1 and DT 2), Cefoxitin resistance (FOXS) and high level gentomycin resistance (GEN 500µg/ml). Each *S. aureus* isolate was grown on BPA agar at 35℃ for 24 hours. One typical colony from BPA agar were added to the demineralized water (TREK Diagnostic Systems, Cleveland, Ohio) and adjusted to a suitable turbidity using the Sensititre system (the valid turbidity present as a green light on the system). Then 10 µl suspension was diluted into a sterile Sensititre adjusted Mueller-Hinton broth (TREK Diagnostic Systems,

Cleveland, Ohio) to about $3x10^5$ CFU/ml. Then an autoinoculator (TREK Diagnostic Systems, Cleveland, Ohio) was used to dispense 50 µl of cell suspension into each well on a Sensititre 96-well GP panel which has been pre-loaded with 22 different antimicrobials. Then the 96-well panel was sealed and incubated at 35℃ for 24 hours. The MIC results were recorded manually based on the growth of bacteria on each well.

Characterization of MRSA

For further characterization of recovered MRSA strains, the tests for sub-typing MRSA strains were performed including PFGE, *SCCmec* typing, PVL gene detection and other virulence factors detection.

Pulsed Field Gel Electrophoresis (PFGE)

After the confirmed MRSA strain grown on BPA at 35°C for 18-24 hours, a single colony was inoculated into BHI broth at 35-37°C for 20-24 hours. Then 1.8% SeaKem Gold agarose was prepared in TE buffer and the gel was placed in a 65°C waterbath. The incubated BHI broth suspension was adjust by turbidity on a MicroScan reader to a range of 1.1 to 1.3. Then 200 µl of the cell suspension was transfer to a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 3-5 minutes. The supernatant was disposed and the pellet was resuspended in 300 µl TE buffer. After the tube was equilibrated in 37°C waterbath for15 minutes, 4 µl lysostaphin was added into the cell suspension carefully but quickly, followed by mixing 300 µl of the pre-prepared 1.8% agarose gel to the suspension gently. The

23

mixture then was dispensed into a plug mold and solidified at a refrigerator for 10 minutes. After being removed from the plug mold, the plug was placed into a tube containing at least 3 ml EC lysis buffer for cell lysis at 37°C for at least 4 hours, the EC lysis buffer was poured out and the tube with plug was replaced with 4 ml TE buffer and placed on a rotator for 30 minutes washing. The washing than was repeated 3 more times and a 1mm X 10 mm size fragment was cut from a prepared *S. aureus* plug and placed in a 1.5 ml centrifuge tube with 150 μl of 1 X NEB 4 buffer (New England Biolabs, Ipswich, MA). After the tube was equilibrated at 25°C for 15 min, 147 μl of 1X NEB 4 buffer with 3 μl *Sma*I restriction enzyme (New England Biolabs, Ipswich, MA) was replaced into the centrifuge tube and incubated at 25°C for at least 4 hours. The *Salmonella braenderup* strain H9812 was used as a molecular weight standard ladder by using *Xba*I restriction enzyme to cut at 37°C for at least 4 hours. During the same time, 1.5 g SeaKem Gold Agarose (Lonza, Rockland, ME) was weighted out and placed in a 500 ml Erlenmeyer flask, and 150 ml of 0.5 X TBE Buffer solution was added. Microwave was used to dissolve the agarose for 2-3 min. And then the 1% gel was placed in 55°C water bath until ready to pour. The restriction cut plug was gently picked out from the 1.5 ml centrifuge tube and loaded onto the bottom of comb, including H9812 standards on the first, middle and last places on the comb. Then, the 1% gel from 55°C water bath was poured to the gel mold. After about 30 min solidification, the Bio Rad CHEF III system (Bio Rad, Hercules, CA) was used to run the PFGE gel at the following condition: 200 Volts, 14°C, initial switch time 5 seconds, final switch time 40 seconds and running time 21 hours. After running, the gel was stained with ethidium bromide for 15

minutes and was destained for 30 minutes in distilled water for 3 times. Photograph was obtained by using BIO-RAD Universal Hood II imaging system (Bio Rad, Hercules, CA) under UV light.

PCR for *SCCmec* typing

A multiplex PCR which contained 9 pairs of primers was performed for *SCCmec* typing, including 8 pairs of primers that target 8 different *SCCmec* types and 1 pair of primers that target *mec*A gene (Table 2-1). The PCR were performed at a 25 μl reaction solution containing 2 μl of DNA template, 2.5 μl of 10×PCR buffer, 2 μl of a 1.25 mM mixture of deoxynucleoside triphophate, 4 μl of 25mM $MgCl_2$, and 0.25 μl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 0.5 μl of mixed oligonucleotide primers (Invitrogen, Carlsbad, CA). Thermocycling protocol included an initial denaturation at 94 °C for 10 min, followed by 10 cycles of denaturation (94 °C for 45 s), annealing (65 °C for 45 s), and extension (72 °C for 1.5 min), and another 25 cycles of denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 1.5 min), ending with a extension at 72 °C for 10 min. 4 μl PCR products were mixed with 1 μl loading buffer and added to a 2% (wt/vol) agarose gel, and an electrophoresis was run at 100mV for 40 min. Gels were then stained with ethidium bromide and the DNA bands were visualized and photographed under UV..

PCR for detecting SPA gene

A single PCR targeting the SPA gene which encode the *S. aureus* specific virulence factor proteinA were performed at a 25 μl reaction solution containing 2 μl of DNA template, 2.5 μl of 10×PCR buffer, 2 μl of a 1.25 mM mixture of deoxynucleoside triphophate, 4 μl of 25mM $MgCl_2$, and 0.25 μl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 1 μl (25pmol) of each oligonucleotide primer (Invitrogen, Carlsbad, CA). Thermocycling protocol included an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation (94 °C for 25 s), annealing (48 °C for 40 s), and extension (72 °C for 1 min), ending with a extension at 72 °C for 10 min. 4 μl PCR products were mixed with 1 μl loading buffer and added to a 2% (wt/vol) agarose gel, and an electrophoresis was run at 100mV for 40 min. Gels were then stained with ethidium bromide and the DNA bands were visualized and photographed under UV illumination. MRSA strain ATCC43300 containing both *nuc* and *mec*A genes was used as the positive control.

Multiplex PCR for detecting PVL and *hlg* genes

A multiplex PCR targeting the PVL gene which encode the Panton-Valentine Leukocidin and hlg gene which encoded the γ-hemolysin were performed at a 25 μl reaction solution containing 2 μl of DNA template, 2.5 μl of 10×PCR buffer, 2 μl of a 1.25 mM mixture of deoxynucleoside triphosphate, 4 μl of 25mM $MgCl_2$, and 0.25 μl of 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ) and 0.5 μl (25pmol) of each hlg oligonucleotide primer and 0.5 (12.5pmol) of each luk-PV oligonucleotide primer (Invitrogen, Carlsbad, CA). Thermocycling protocol

included an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 1 min), ending with a extension at 72 °C for 10 min. 4 μl PCR products were mixed with 1 μl loading buffer and added to a 2% (wt/vol) agarose gel, and an electrophoresis was run at 100mV for 40 min. Gels were then stained with ethidium bromide and the DNA bands were visualized and photographed under UV illumination. MRSA strain ATCC43300 containing both *nuc* and *mec*A genes was used as the positive control.

Statistical analysis

Statistical analyses were performed in SPSS v13.0 (SPSS, Inc. Chicago, Illinois, USA). χ^2 test was performed to compare three or more groups of categorical data, and Fisher's exact test was performed to compare two sites of data. P value < 0.05 has been considered as a significant difference.

Table 2-2. Oligonucleotide primers used for PCR assays

Primer Name	Primer Sequence	Specificity	Product Size (bp)	Reference
*mec*A-F	AAAATCGATGGTAAAGGTTGGC	*mec*A	533	K. Murakami et al. 1991
*mec*A-R	AGTTCTGCAGTACCGGATTTGC			
nuc-1	GCGATTGATGGTGATACGGTT	*nuc*	270	O. G Brakstad et al. 1992
nuc-2	AGCCAAGCCTTGACGAACTAAAGC			
*mecA*2-F	CTCAGGTACTGCTATCCACC	*mec*A	448	G. Sakoulas et al. 2001
*mecA*2-R	CACTTGGTATATCTTCACC			
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	*SCCmec* I	613	K. Zhang et al. 2005
Type I-R	GTTCTCTCATAGTATGACGTCC			
Type II-F	CGTTGAAGATGATGAAGCG	*SCCmec* II	398	K. Zhang et al. 2005
Type II-R	CGAAATCAATGGTTAATGGACC			
Type III-F	CCATATTGTGTACGATGCG	*SCCmec* III	280	K. Zhang et al. 2005
Type III-R	CCTTAGTTGTCGTAACAGATCG			
Type IVa-F	GCCTTATTCGAAGAAACCG	*SCCmec* IVa	776	K. Zhang et al. 2005
Type IVa-R	CTACTCTTCTGAAAAGCGTCG			
Type IVb-F	TCTGGAATTACTTCAGCTGC	*SCCmec* IVb	493	K. Zhang et al. 2005
Type IVb-R	AAACAATATTGCTCTCCCTC			
Type IVc-F	ACAATATTTGTATTATCGGAGAGC	*SCCmec* Ivc	200	K. Zhang et al. 2005
Type IVc-R	TTGGTATGAGGTATTGCTGG			
Type IVd-F5	CTCAAAATACGGACCCCAATACA	*SCCmec* Ivd	881	K. Zhang et al. 2005
Type IVd-R6	TGCTCCAGTAATTGCTAAAG			
Type V-F	GAACATTGTTACTTAAATGAGCG	*SCCmec* V	325	K. Zhang et al. 2005
Type V-R	TGAAAGTTGTACCCTTGACACC			
*Mec*A147-F	GTGAAGATATACCAAGTGATT	*mec*A	147	K. Zhang et al. 2005
*Mec*A147-R	ATGCGCTATAGATTGAAAGGAT			
luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	PVL	433	Gerard Lina et al. 1999
luk-PV-2	GCATCAASTGTATTGGATAGCAAAAGC			
hlg-1	GCCAATCCGTTATTAGAAAATGC	hemolysin	937	Gerard Lina et al. 1999
hlg-2	CCATAGACGTAGCAACGGAT			
spa-1	TCAAGCACCAAAAGAGGAAGA	Protein A	about 263	J. Walker et al. 1998
spa-2	ACGACATGTACTCCGTTGCCG			

Chapter 3: RESULTS

<u>Prevalence of *S. aureus* and MRSA in ground meat products</u>

A total of 480 ground meat samples, including 231 ground pork and 249 ground beef, were collected from three local retail stores which represented three different local food supply chains in the Washington DC area from March 2009 to March 2010. One hundred sixty two samples were collected from store A, 160 from store B and 158 from store C. *S. aureus* were recovered from 202 (42.08%) samples, including 117 (50.65%) ground pork and 85 (34.14%) ground beef, and ground pork has a significant higher level of *S. aureus* contamination than ground beef (Table 3-1). In different seasons, the prevalence of *S. aureus* was significantly different in both ground beef and ground pork samples. In collected ground pork samples, *S. aureus* was recovered from 43 (36.75%) in Spring (March 2009 to May 2009), 30 (25.64%) in Summer (June 2009 to August 2009), 17 (14.53%) in Fall (September 2009 to November 2009) and 27 (20.08%) in Winter (December 2009 to March 2010) (Figure 3-1). For ground beef, *S. aureus* was recovered from 33 (38.82%) samples in Spring, 16 (18.82%) in Summer, 15 (17.65%) in Fall and 21 (24.71%) in Winter (Figure 3-1). During the one year sampling, 74 (45.68%) samples of chain A had *S. aureus* isolates presented, and 56 (35%) in chain B, 72 (45.57%) in chain C. Chain B had a significant lower level of *S. aureus* contamination when comparing with chain C (Table 3-2). In addition, because of the variety of the lean and fat ratio in ground beef, the *S. aureus* positive rate that related with the percentage of fat in ground beef products was also evaluated by 10 different fat contents from 4% to 27% (Figure 3-2). Among 202 (42.08%) *S. aureus* positive samples, only one MRSA strain was

isolated from a ground beef from chain B, which we numbered as MRSA285 (Table

3-1), and confirmed by PCR for *mec*A and *nuc* genes. (Figure 3-3)

Table 3-1. Prevalence of *S. aureus* and MRSA in ground meats

Meat type	No. of samples	No. (%) of *S. aureus* including MRSA*	MRSA alone[a]
Ground Beef	249	85 (34.14)	1 (0.4)
Ground Pork	231	117 (50.65)	0 (0)
Total[b]	480	202 (42.08)	1 (0.21)

a. MRSA strain was isolated from the grocery store in chain B in Sep. 24. 2009
b. considering ground beef and ground pork as one group
*. Fisher's exact test indicates the prevalence of *S. aureus* in ground beef and ground pork is significantly different. (P<0.05)

Table 3-2. The prevalence of *S. aureus* in the food supply chains

Food Chains[a]	No. of samples	Total No. (%) of *S. aureus* +	No. (%) of *S. aureus* + in ground Beef[b]	No. (%) of *S. aureus* + in ground Pork[c]
Chain A	162	74 (45.68)	32 (38.55)	42 (52.5)
Chain B	160	56 (35)	24 (28.56)	32 (41.03)
Chain C	158	72 (45.57)	29 (34.52)	43 (56.58)

a. Fisher's exact test indicates the prevalence of *S. aureus* is significantly different between chain B and chain C. (P<0.05)
b.c. The prevalence of ground beef and ground pork has no significant difference in Chain A and Chain C, but is significantly different in Chain C. (P<0.05)
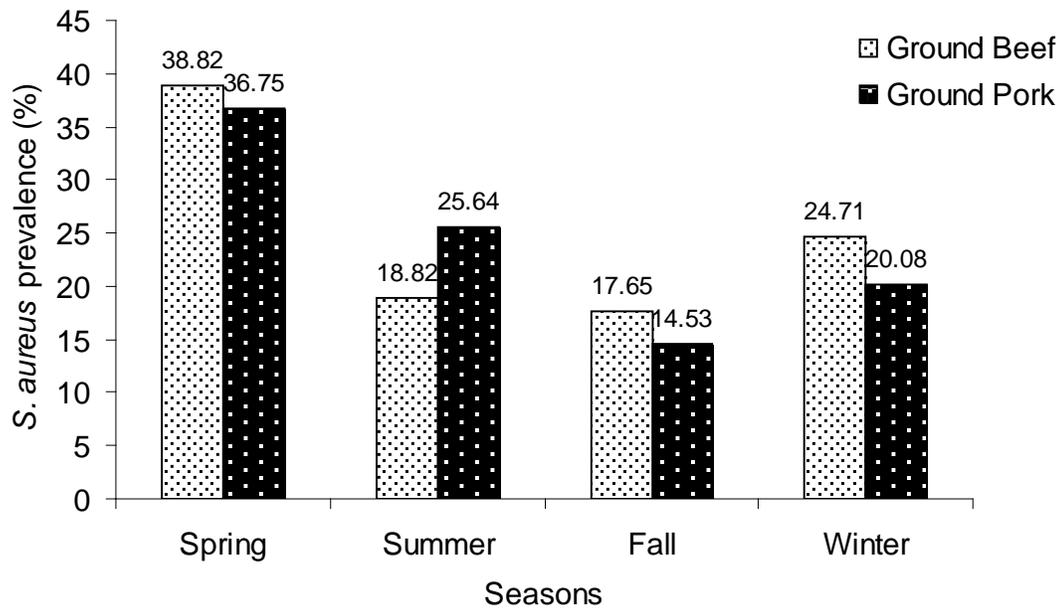
Figure 3-1. Prevalence of *S. aureus* associated with seasons. The seasonal changes were investigated based on the sampling date information. Spring season began from March 2009 to May 2009, Summer period was defined as June 2009 to August 2009, Fall season was defined as September 2009 to November 2009 and Winter was defined as December 2009 to March 2010. Statistical analysis was performed to evaluate the difference between different seasons. χ^2 test indicated that season has a significant effect on *S. aureus* prevalence in both ground beef and ground pork (P<0.05).The data represent the percentage of samples in ground beef and ground pork.
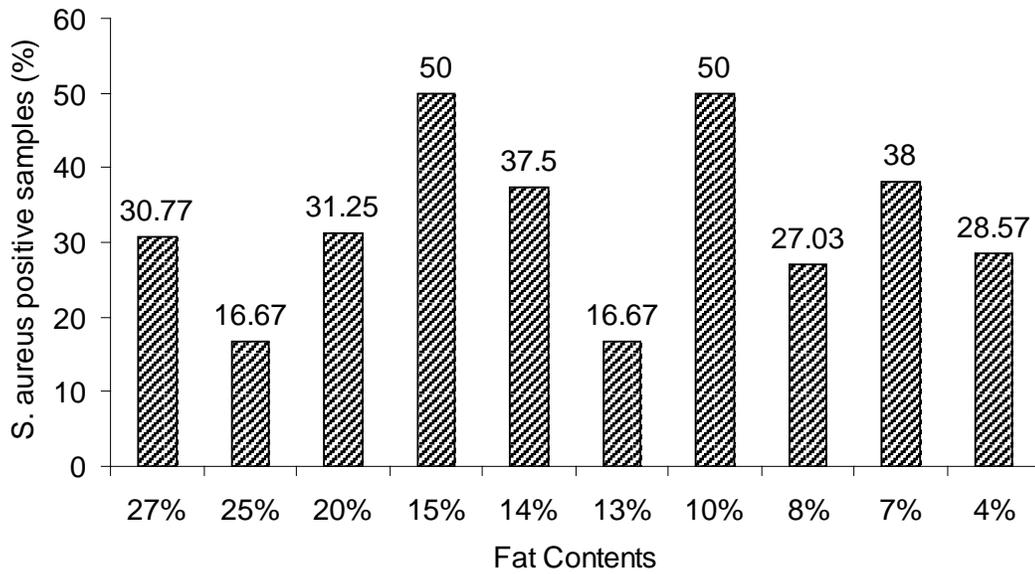
Figure 3-2. Presence of *S. aureus* in ground beef with different fat contents. The samples were collected randomly from each retail store with 10 different fat and lean ratio, from 27% to 4%. The data represent the change of *S. aureus* contamination with the change of fat/lean ratio.
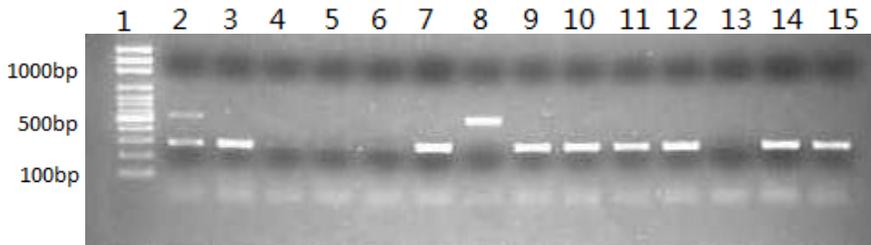

Figure 3-3. Multiplex PCR results of *S. aureus* and MRSA isolates. The size of *nuc* and *mec*A are 270 and 533bp, respectively. Line 1, 100bp DNA ladder; Line 2, Positive control of MRSA strain; Line 3,7,9,10,11,12,14,15, *S. aureus* strains; Line 8, non-*S. aureus* located with *mec*A antimicrobial resistant gene.

Antimicrobial resistance patterns of *S. aureus* and MRSA

After *S. aureus* and MRSA isolates were confirmed by PCR and biochemical tests, 525 *S. aureus* isolates, including 3 MRSA isolates, from 202 retail meat samples were examined for antimicrobial susceptibility using Sensititre MIC susceptibility system. In our study, antimicrobial resistant *S. aureus* has been found for 13 different antimicrobials, and the most common resistance within 525 *S. aureus* isolates are TET, PEN, ERY and CLI resistance. Especially for TET, more than half (54.46%) of the *S. aureus* from contaminated meat samples show resistance to this drug. In addition, there are 13 samples exhibited an inducible clindamycin resistance (Table 3-3). For CHL, GEN, AMP, LEVO, CIP, SYN and TGC, although a few resistant isolates are identified, the MIC results show that antimicrobial resistance is not yet common in recovered *S. aureus*. For CHL, 77.52% *S. aureus* isolates have the ability to resist to 8 µl/ml CHL and 21.33% could tolerant to 16 µl/ml CHL. However, only 1 *S. aureus* strain can resist to the breakpoint concentration as CHL >16 µl/ml (Figure 3-4). For GEN, although there were two *S. aureus* isolates can be classified as GEN resistant *S. aureus*, 99.05% isolates are susceptible to this drug. (Figure 3-5). Most recovered *S. aureus* are still susceptible to AMP, but more than half of these isolates have certain levels of resistance and 10 (1.9%) AMP resistant *S. aureus* was identified (Figure 3-6). Penicillin as the first introduced antimicrobial to cure *S. aureus* infections still remained effective for 41.14% *S. aureus* isolates recovered from meat samples, but there were 33 (6.48%) isolates are penicillin resistant and 68 (12.95%) isolates have the ability to resist to 8 µl/ml PEN (Figure 3-7). Although the resistant *S. aureus* was found for SYN, CLI and CIP, the majority of

recovered *S. aureus* were still susceptible to these three drugs (SYN susceptible 97.33%, CLI susceptible 95.24%, CIP susceptible 99.24%). ERY has 32 (6.1%) resistant strains identified, but 86.67% recovered isolates can only resist to 0.5 µl/ml ERY (Figure 3-8). TGC has 42.1% of total *S. aureus* isolates can resist to 0.25 µl/ml concentration, and 26.1% isolates have the ability to resistant near to the 0.5 µl/ml break point (Figure 3-9). The MIC result for TET was very unique. Almost all isolates presented only two level of susceptibility, either resistance (51.62%) or susceptible (48.19%) (Figure 3-10). Vancomycin, as the last choice for curing MRSA infection, is still effective for all *S. aureus* strains, no resistance has been observed. However, 84.57% isolates show resistance to 1 µl/ml vancomycin (Figure 3-11). The only oxacillin resistant isolates were the MRSA strain which comes from the only MRSA positive sample, and the rest of *S. aureus* strains are still susceptible for oxacillin (Figure 3-12). This only 1 MRSA strain shown a multi-drug resistant pattern to 8 different antimicrobials (Table 3-4), including ERY, OXA, AMP, FOXS, PEN, LEVO, CIP and MXF. For other non-MRSA *S. aureus* isolates, multidrug resistant were also exhibited. 38 (7.24%) *S. aureus* isolates can resist to 2 different drugs simultaneously and 13 (2.48%) *S. aureus* isolates have the ability to resist to 3 antimicrobial which have been classified as multidrug resistant *S. aureus* (Table 3-4).

Table 3-3. Antimicrobial resistant profiles for *S. aureus* positive samples including

MRSA

Antimicrobials [a]	Samples (No.)[b]	Samples (%)
CHL	1	0.5
ERY	16	7.92
CLI	10	4.95
OXA	1	0.5
DT	13	6.44
GEN	2	1
AMP	7	3.47
FOXS	1	0.5
PEN	23	11.39
LEVO	1	0.5
CIP	1	0.5
SYN	1	0.5
TGC	4	1.98
TET	110	54.46

a. CHL Chloramphenicol, ERY Erythromycin, CLI Clindamycin, OXA Oxacillin+2%NaCl, DT inducible Clindamycin, GEN Gentamicin, AMP Ampicillin, FOXS Cefoxitin, PEN Penicillin, LEVO Levofloxacin, CIP Ciprofloxacin, SYN Quinupristin/dalfopristin, TGC Tigecycline, TET Tetracycline
b. For each sample, up to 3 confirmed *S. aureus* or MRSA strains have been tested. The sample was recorded when at least one strain was resistant to certain drugs

Table 3-4. Multi-drug resistant patterns for *S. aureus* isolates including MRSA

No. of drugs	0	1	2	3[a]	4	5	6	7	8[b]
% of isolates	46.1	43.62	7.24	2.48	0	0	0	0	0.57
No. of isolates	242	229	38	13	0	0	0	0	3

a. multi-drug resistant *S. aureus* was defined as the strain has the ability to resist to 3 or more antimicrobials simultaneously.
b. The MRSA strain shown resistance to ERY, OXA, AMP, FOXS, PEN, LEVO, CIP and MXF.
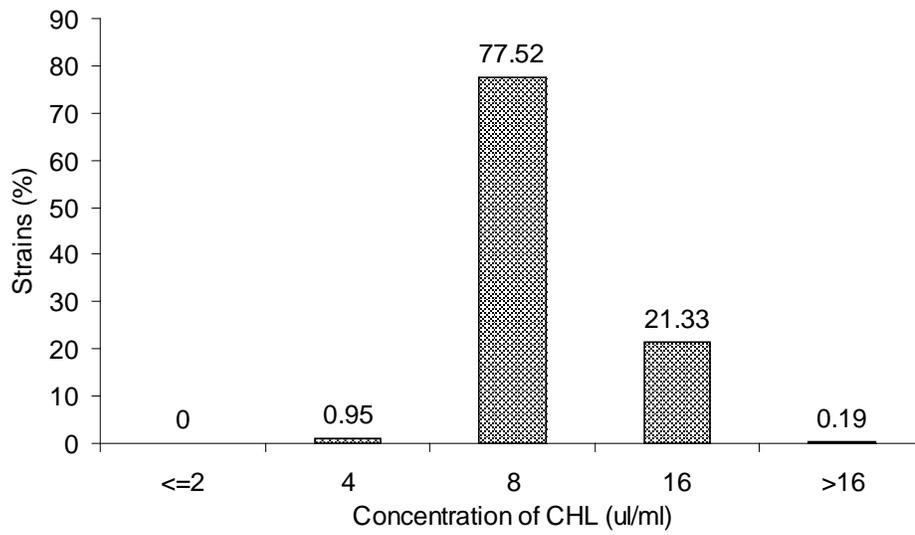
Figure 3-4. MICs of Chloramphenicol (CHL) among *S. aureus*.
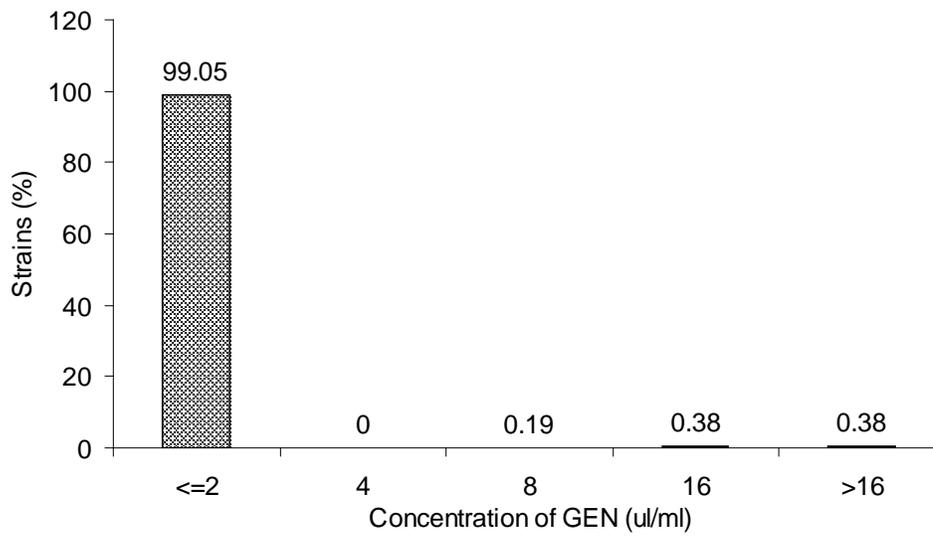

Figure 3-5. MICs of Gentamicin (GEN) among *S. aureus* .
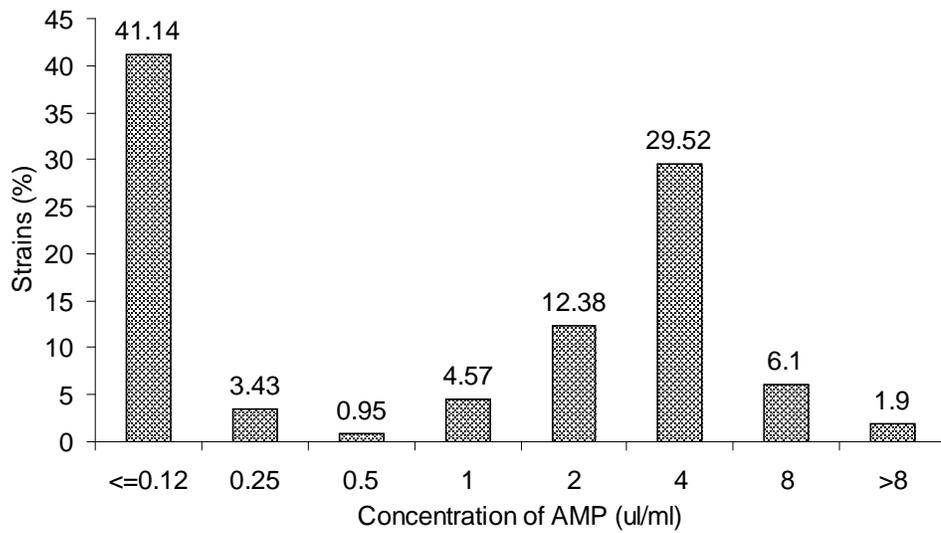
Figure 3-6. MICs of Ampicillin (AMP) among *S. aureus* .
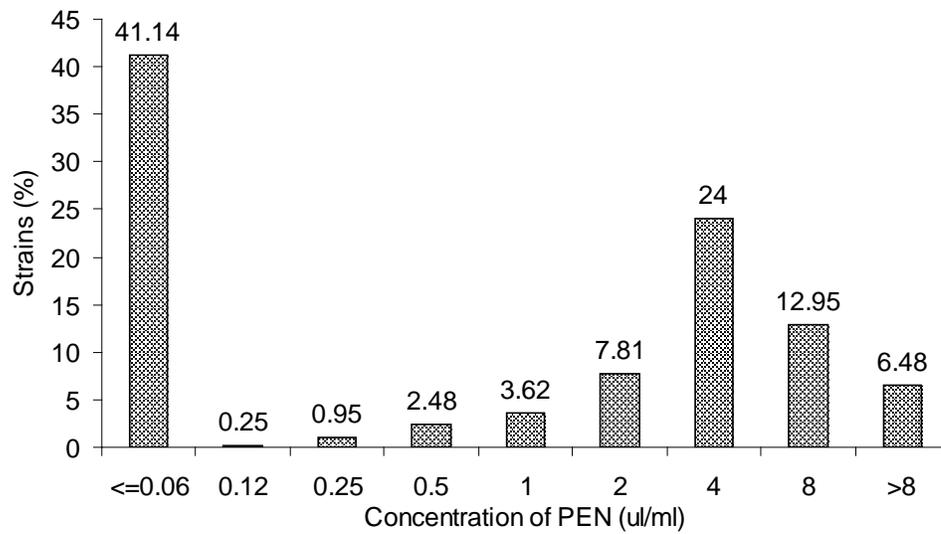
Figure 3-7. MICs of Penicillin (PEN) among *S. aureus*.
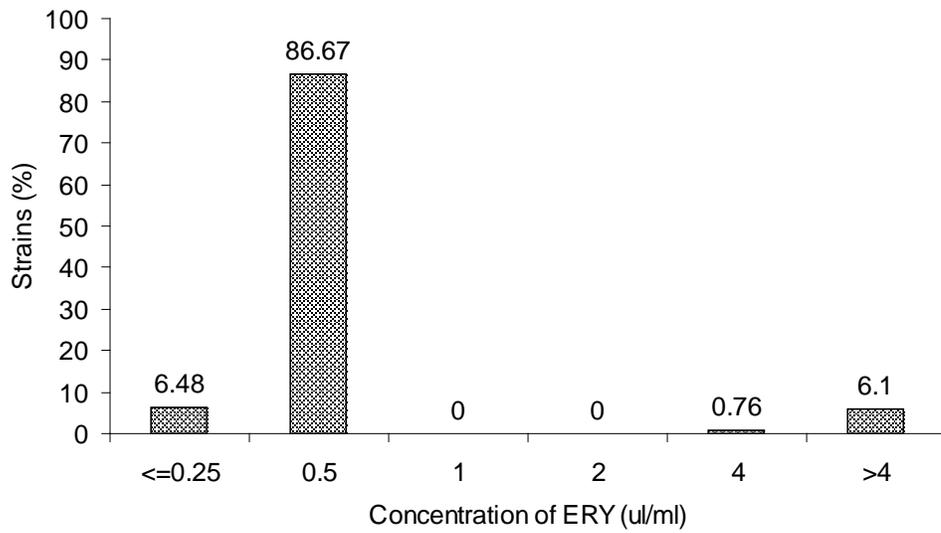
Figure 3-8. MICs of Erythromycin (ERY) among *S. aureus*.
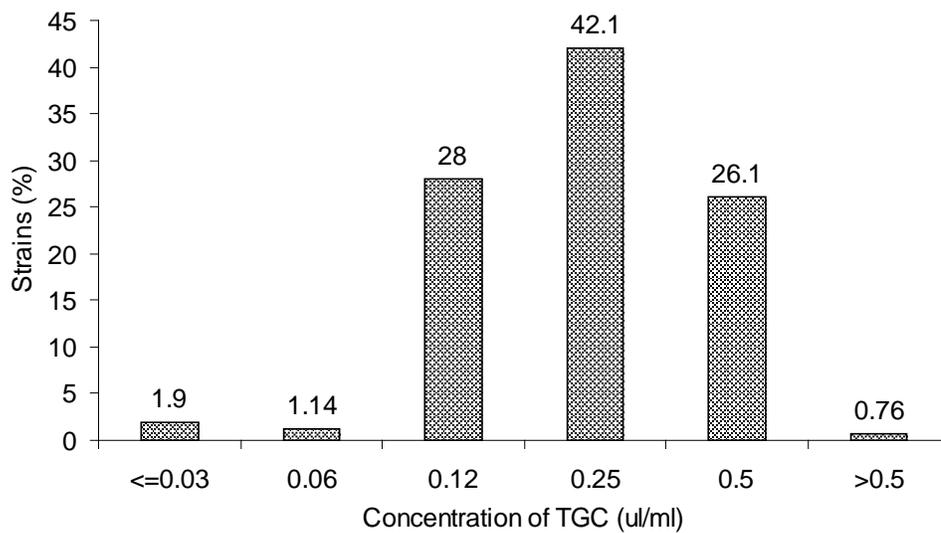
Figure 3-9. MICs of Tigecycline (TGC) among *S. aureus*
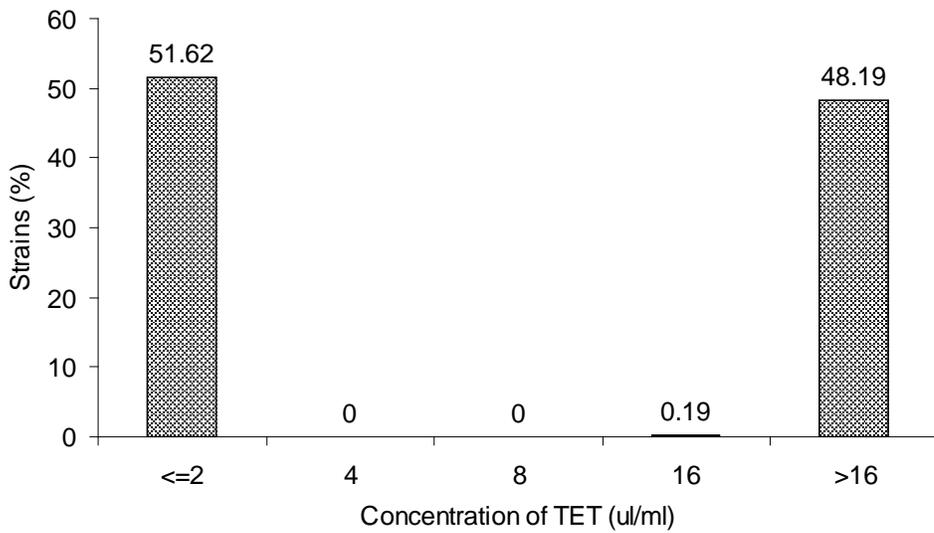
Figure 3-10. MICs of Tetracycline (TET) among *S. aureus*
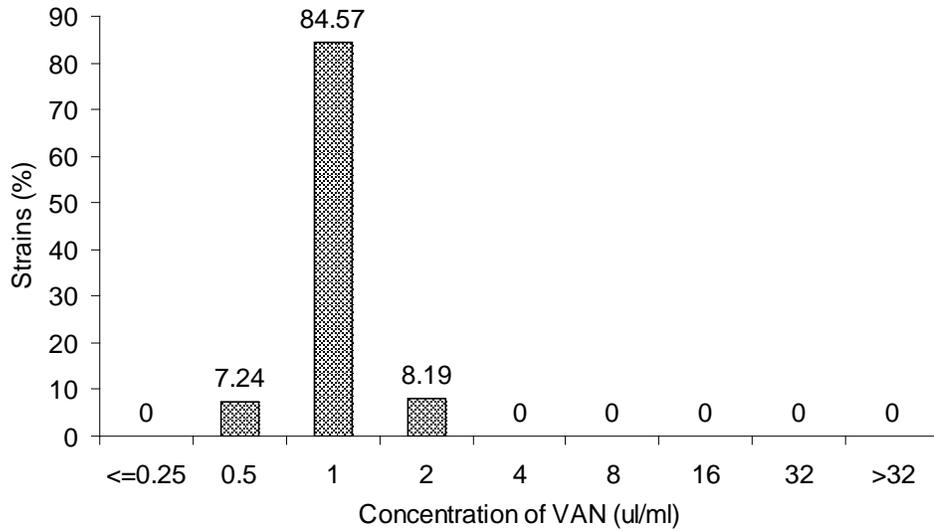
Figure 3-11. MICs of Vancomycin (VAN) among *S. aureus*
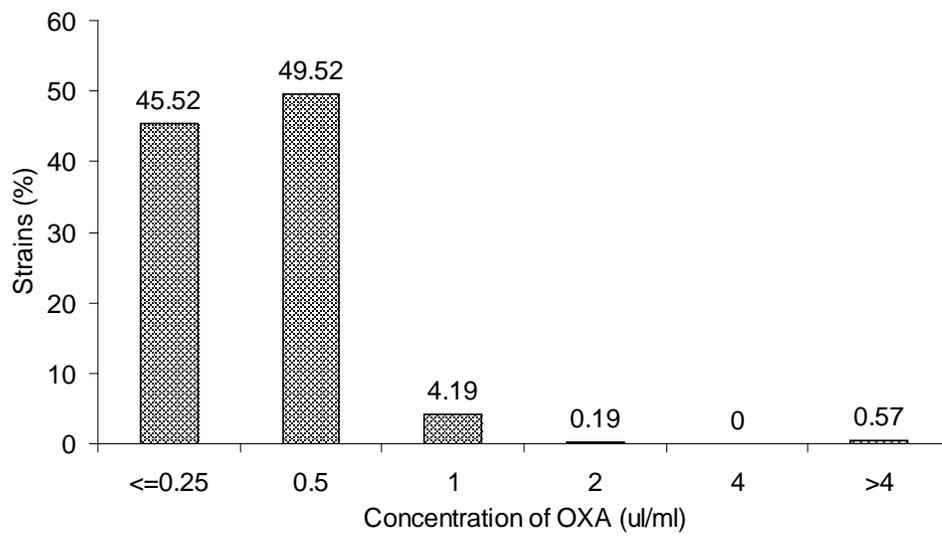
Figure 3-12. MICs of Oxacillin (OXA) among *S. aureus*

MRSA Sub-typing and virulence genes detection

For better understanding of the recovered MRSA285 strain, sub-typing was performed using PFGE and *SCCmec* chromosome cassette typing. In addition, several virulence genes were also performed to help characterize this MRSA strain. Compared with standard strains, PFGE results indicated all five isolates showed an identical profile to USA300 subtype (Figure 3-13). This is one of the most common Community Associated MRSA strains. For *SCCmec* typing, the PCR results showed that *mec*A, which indicated its ability to resist to β-lactams. However, no bands at expected positions for *SCCmec* on the gel (Figure 3-14), which indicted the lack of eight typical *SCCmec* types. The results for detecting virulence genes showed the presence of PVL and SPA genes (Figure 3-15). In addition, PVL gene of the MRSA285 strain indicated it belonged to CA-MRSA.
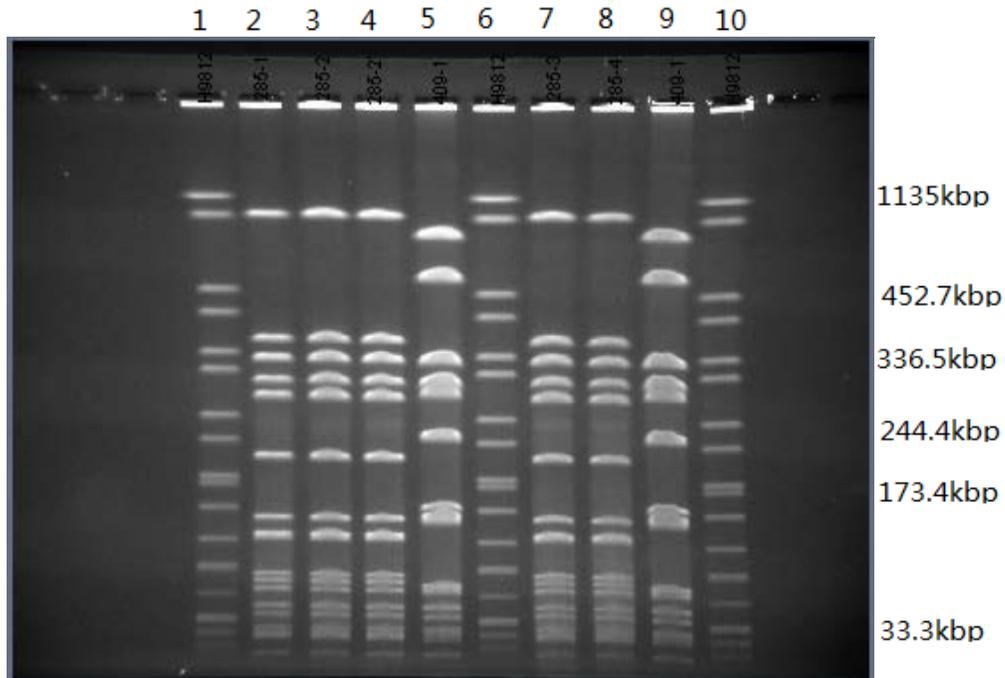
Figure 3-13. Pulsed-field Gel Electrophoresis (PFGE) for MRSA. The PFGE typing followed the CDC PulseNet protocol of molecular typing of oxacillin-resistant *Staphylococcus aureus* by PFGE. 5 single colonies were picked out from the MRSA positive sample. After confirmation, these 5 single colonies were inoculated in BHI broth and followed by PFGE test. This figure represents that PFGE patterns on the gel under UV light. From left to right: Line 1, 6 and 10, H9812 Ladder. Line 2, 3, 4, 7 and 8, MRSA isolates from ground beef in this study (USA300). Line 5 and 9, MRSA isolates from previous study (USA100).
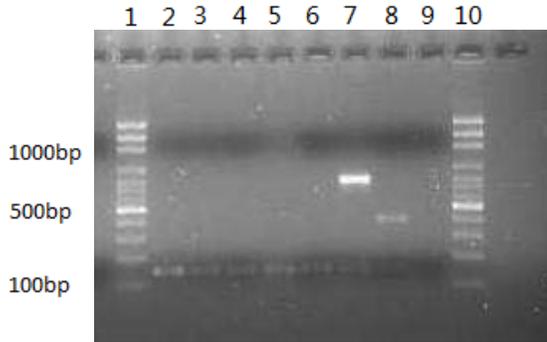
Figure 3-14. PCR for *SCCmec* typing. 5 MRSA isolates from the same MRSA positive sample were cultured on BPA for 24 hours at 35°C. After the genomic DNA have been extracted by boiling at 100°C for 15 min, a multiplex PCR with 9 sets of primers was performed for these 5 isolates. Each set of primer amplifies one *SCCmec* type; and1 set of primer which amplifies the *mec*A gene has also been included. From left to right: Line 1 and 10, 100bp DNA ladder. Line 2-6, 5 repeats of MRSA strain from this study. Line 7, MRSA strain from previous study in UMD. Line 8, ATCC4300 Clinical strains. Line 9, Negative control.
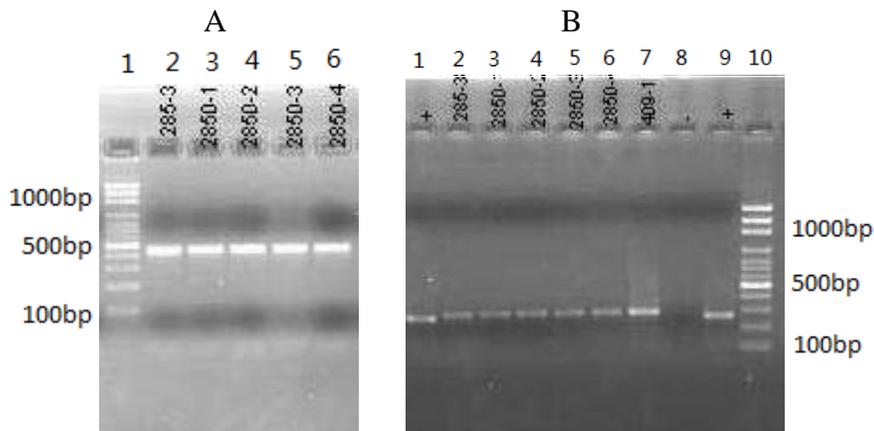
Figure 3-15. PCR for detecting PVL and SPA genes. 5 MRSA isolates from the same MRSA positive sample were cultured on BPA for 24 hours at 35°C. After the genomic DNA have been extracted by boiling at 100°C for 15 min, PCR was performed for these 5 isolates to detect PVL gene (A) and SPA gene (B). From left to right: (A): Line 1, 100bp DNA ladder. Line 2-6, MRSA strain from this study. (B): Line 10, 100bp DNA ladder. Line 2-6, MRSA strain from this study. Line 1 and 9, positive control. Line 8, negative control.

43

Chapter 4: DISCUSSION

S. aureus as a ubiquitous bacterium is the leading cause of superficial infection at the clinical environment for decades and also considered as the third most important cause of reported food-borne illnesses in the world (92). As the result of the widely use of antimicrobials, MRSA caused the increase of hospital-acquired infection (97). And in the past 20 years, the rising number of infections out of hospital environment caused by CA- MRSA indicated the spreading of MRSA from hospital to our daily life. Since a large number of animals can be colonized with *S. aureus* and the use of antimicrobial agents in veterinary medicine is very common, the increase of antimicrobial resistance was observed and MRSA strains were isolated from several common food animals, including cattle, pigs, chicken (54, 98-100). During slaughtering of food animals which had been colonized with MRSA, food products may eventually be contaminated with MRSA. In addition, inappropriate handling of food products or contact with MRSA carrier may also cause the food contaminated with MRSA (101-102). Under these circumstances, food products, especially fresh raw meats have the potential to transfer *S. aureus* or MRSA from animals to humans.

In this study, 480 raw ground meat samples collected from March 2009 to March 2010 were examined for *S. aureus*. Approximately 42% (202) of the samples were positive of *S. aureus*. The positive rate for ground beef was 34.14% and for ground pork was 50.65%. Compared with our previous study in 2008, which showed 27.8% of ground beef and 11.7% of ground pork samples were positive of *S. aureus*, the increase of positive rate in the products may be due to the difference in isolation

methods used. In this study, a selective enrichment broth was added to increase the number of the target bacterium before using selective media. Researchers in Netherland reported the prevalence of *S. aureus* was 33.33% in beef and 45.31% in pork (103). Pesavento et al. reported 29.41% of beef, 15.15% of pork meat were positive in Italy (104). Another study conducted in Italy found *S. aureus* in 31.2% ground meat and 26.1% in fresh meat (92). In US, a study by Pu et al. reported *S. aureus* in 20% beef samples and 45.6% in pork samples in Louisiana (93). Many factors, such as sampling, isolation methods and geological locations, may contribute to the variety of positive percentages and also make the comparisons between different studies difficult. The lacking of a well established standard isolation method also makes the evaluation of our methodology very intricate. For the study performed in Louisiana which used a protocol similar to ours, the higher prevalence rate our study reported may attribute to the difference of sample types. Instead of taking beef steaks and pork chops, we collected ground beef and pork products. Based on the facts of raw meat products manufacturing, ground meat generally mixed by different parts of food animals and more than one single food animal for each lot, which indicated that ground meat has the higher probability to be contaminated during processing if food animals carried *S. aureus*. From the existing research data, although direct comparison is not suitable between these studies, all results indicated that *S. aureus* is not a rare bacterium in various food samples. Our data also showed a similar prevalence rate of *S. aureus* in ground beef and pork from different retail stores under three chain managements. In addition, the data, which analyzed the association of different seasons on *S. aureus* contamination rate, indicated that season

was a significant factor that affected *S. aureus* contamination rate in Spring (March to May). More *S. aureus* contaminated samples were identified in Spring and Winter. Comparing with one clinical study, Skull et al. reported a peak incidence of *S. aureus* infection in hospital occurred between October to March (105). Interestingly, the seasons with the highest recovery rate of *S. aureus* in meat samples matched with the peak season of *S. aureus* infections in hospitals. This may imply the possible relationship between *S. aureus* carriers and contaminated food products. Because a higher *S. aureus* infection indicated a higher *S. aureus* colonization in human, this may eventually lead to a higher contamination rate in food due to the contact of food and handlers. This corresponding data in our study may also indirectly prove that food handler is a majority contamination sources for *S. aureus* in food. Additionally, an irregular change between *S. aureus* recovering rate and the various percent of fat in ground beef seemed that the contamination rate of *S. aureus* had no relation to the fat contents in food product.

Among the 202 *S. aureus* positive samples, only one ground beef was contaminated with MRSA. The prevalence of MRSA in this study was 0.21% (0% in ground pork, and 0.4% in ground beef). MRSA was detected in 3.3% beef samples and in 5.6% pork in Louisiana (93). An earlier study in our lab also identified one MRSA from a ground pork sample of 694 meat samples (0.14%) collected. Two independent studies in Netherlands reported MRSA in 3.13% pork samples, 0% beef samples (103) and in 10.6% beef and 10.7% pork samples (101). Again, various factors could contribute to the differences in these studies, directly comparing the existing data is inappropriate. However, based on these studies, although MRSA was

presented in the tested food products, the positive rate remained low. In our study, we also tried to add colony hybridization in our isolation method to increase the probability of isolating MRSA, the positive rate for MRSA still remained to be a very low level. On the other hand, the presence of MRSA in various meat products demonstrated that food has the potential to serve as a transmission vehicle to spread MRSA in our community.

One dilemma for studying MRSA is the classification of MRSA and MSSA. Because of the changing concept on MRSA, there are no unified standards to evaluate if one *S. aureus* strain is MRSA or not. The questions, such as which antimicrobials should be used as selective agents to isolate MRSA, should antimicrobial resistance results be used as the standard to separate MRSA from MSSA, or if molecular based detection methods targeting specific genes are the better distinction method, are still under debate. Pereira reported that in Portugal 38% of *S. aureus* isolates were oxacillin resistant but only 0.68% of the isolates contain *mec*A gene, which was recognized as the most important gene marker to differentiate MRSA (56). Similar situation occurred in Italy, a study found 30% of *S. aureus* positive beef samples and 10% of *S. aureus* positive pork samples were resistant to oxacillin, but none of *S. aureus* isolate resistant to methicillin (104). This dilemma makes the studies of MRSA difficult to evaluate, and the comparison of different studies from various researchers or locations also become very complex. In our study, MRSA identified as *S. aureus* with oxacillin resistance containing *mec*A gene.

In this study, the AST results for *S. aureus* isolates show that the antimicrobial with the highest resistant rate was TET (54.45%), followed by PEN (11.39%) and

ERY (7.92%). The research data from other studies in different countries showed various patterns on antimicrobial resistance. The study in Portugal reported 0.7% resistance in TET, 73% in PEN and 5% in ERY. In addition, 70% *S. aureus* were resistant to AMP, which only had 3.47% resistance in our study (56). The AST study on *S. aureus* in Italy found 19.04% *S. aureus* isolates from meat samples were resistant to TET, 16.66% resistant to PEN, and 42.86% to AMP (104). A study in Turkey also reported 53.8% PEN resistance and 7.5% ERY resistance (106). As each independent study used a different AST protocol, data comparison among these studies can be challenging. Antimicrobial resistance patterns of *S. aureus* strains from various locations showed noticeable differences. The strains category and classification may also be different. When comparing the resistance results with the our previous study with 68.5% to TET, 26% to PEN, 8% to ERY and 17% to AMP, the differences were not significant.

In addition to MRSA, multi-drug resistance were also found in *S. aureus* isolates. Pesavento et al. reported that 9.52% of *S. aureus* were resistant to two antimicrobials and 30.95% to three or more antimicrobials (104). However, the only strain resistant to oxacillin was the MRSA strain. It was resistant to eight antimicrobials, including FOXS which is commonly used for MRSA screening. Multiplex PCR identified both *nuc* and *mec*A. It belonged to USA300, A CA-MRSA. The MRSA strain recovered in our previous study belonged to USA100 HA-MRSA based on PFGE and the presence of PVL gene. The study in Louisiana also discovered both USA100 and USA300 MRSA from meat products (93). The occurrence of HA-MRSA strains outside hospitals demonstrated potential widespread

of HA-MRSA from healthcare facility to community. However, as the borders to distinguish HA-MRSA and CA-MRSA become more unclear recently, the evolution relationships need to be investigated.

The absence of *SCCmec* in MRSA is unusual but has been observed in other studies. This could be due to possible new *SCCmec* types as the *SCCmec* typing assay used can only identify eight most common *SCCmec* types. New *SCCmec* types, such as *SCCmec* VI, require designing different PCR assays for detection. Therefore, it is not surprising that the MRSA strain did not belong to the eight most common *SCCmec* types. Further studies are needed for better understanding the revolution of *SCCmec* chromosome cassette.

Chapter 5: CONCLUSION

Staphylococcus aureus, as the major cause of superficial infection and the third most important cause of reported foodborne illnesses around the world, is commonly present in meat products in Washington DC area. Similar findings have been reported by other researchers when survey studies were conducted in other states and countries. Although MRSA is still very rare in the meat products, the existence of MRSA in food samples implied the potential that MRSA can be spread in the community through food.

In addition, a noticeable number of *S. aureus* strains from meat products have developed resistance to various antimicrobials, and multidrug resistant strains are not uncommon. With the current definition of MRSA, methicillin or oxacillin resistant *S. aureus* which is not classified by molecular based tests as MRSA also exist in food products. These *S. aureus* strains have not been well studied and could serve as reservoir of antimicrobial resistance genes and potentially cause infections that can compromise antimicrobial treatment.

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