DETECTION AND MOLECULAR TYPING OF METHICILLIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* (MSSA) AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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Detection and molecular typing of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Methicillin-resistant (MRSA) and multidrug-resistant (MDR) *Staphylococcus aureus*, and the serotype (ST) 398 have been associated with human and livestock infections, being also detected in retail meat. The aim of this study was to determine the prevalence and molecular types of *S. aureus* strains from animals, retail raw meat, deli meat, and humans, determining the genetic similarity between the strains.

A two-step selective enrichment followed by selective plating were used to isolate *S. aureus* from animals (*n*=167), retail raw meat (*n*=145), and deli meat (*n*=46). In addition, *S. aureus* from healthy people (*n*=550) was isolated by culture method. Positive isolates and MRSA isolates from clinical cases (*n*=108) were subjected to multiplex PCR (16S rRNA, *mecA*, and PVL genes), molecular typing and antimicrobial susceptibility testing. In addition, a real-time PCR assay was developed in order to decrease the time of detection of target genes of *S. aureus* in animal and meat samples, comparing the results with the standard culture/PCR method.

The prevalence of *S. aureus* was 34.7% in animals, 47.6% in meat, and 13.0% in deli meat. The *mecA* gene was detected in *S. aureus* isolated from five pork meat samples and exhibited penicillin resistance. The ST398 was found in sheep, pigs, and pork meat. The *S. aureus* nasal carriage in healthy people was 7.6%. A total of 105 MRSA strains (97.2%) from clinical cases harbored the *mecA* gene and 11 (10.2%) the PVL gene. The rate of MDR was 70% in humans. A genetic similarity between strains from animals and meat, and from humans and meat was observed. Total agreement between the culture/PCR method and real-time PCR for detection of *S. aureus* was 68.9 to 97.8% (*k*=0.68-0.88), and the *mecA* gene, 86.7 to 98.7% (*k*=0-0.49).

Therefore, the real-time PCR assay may be recommended as a rapid method for the detection of *S. aureus*, with confirmation of MRSA using the standard culture method. The

presence of emerging *S. aureus* strains in the meat production chain and the genetic similarity between strains of different origin, suggests the contamination of meat, and a potential risk of transmission to humans.

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DEDICATION

To my beloved parents, Gloria and Roberto,

my sister, Claudia,

my brother-in-law, Álvaro,

my lovely nieces, Fernanda, María Ignacia, and Francisca,

without whom none of my success would be possible.

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LIST OF ABBREVIATIONS

AR.............................Antimicrobial-resistant

BORSA......................Borderline oxacillin-resistant *S. aureus*

BP..............................Baird Parker

CA-MRSA.................Community-associated MRSA

CDC...........................Centers for Disease Control and Prevention

CHL...........................Chloramphenicol

CIP.............................Ciprofloxacin

DNA..........................Deoxyribonucleic acid

ERY...........................Erythromycin

FDA...........................Food and Drug Administration

GEN...........................Gentamicin

GPID..........................Gram positive identification

HA-MRSA.................Health care-associated MRSA

KAN...........................Kanamycin

LA-MRSA..................Livestock-associated MRSA

LINC...........................Lincomycin

MDR...........................Multidrug-resistant

MHB...........................Mueller-Hinton broth

MIC.............................Minimum inhibitory concentration

MLST..........................Multilocus sequence typing

mPCR..........................Multiplex PCR

MRSA..........................Methicillin-resistant *S. aureus*

MSSA............................Methicillin-susceptible *S. aureus*

NARMS.........................National Antimicrobial Resistance Monitoring System

QUI................................Quinupristin/dalfopristin

PBP2a............................Penicillin-binding protein 2a

PCR................................Polymerase chain reaction

PEN................................Penicillin

PFGE..............................Pulsed-field gel electrophoresis

$PHMB^+$............................Phenol red mannitol broth + ceftizoxime + aztreonam

PVL.................................Panton-Valentine Leukocidin

RNA................................Ribonucleic acid

rRNA...............................Ribosomal RNA

SB....................................Sheep blood

SCC*mec*............................Staphylococcal cassette chromosome *mec*

ST.....................................Serotype

STR..................................Streptomycin

TET..................................Tetracycline

TSA..................................Trypticase soy agar

USDA...............................U.S. Department of Agriculture

1. GENERAL INTRODUCTION

The emergence and spread of multidrug-resistant (MDR) pathogens have increased public health concerns worldwide. The emergence of antimicrobial-resistant (AR) bacterial strains in animals has been related to the use of antibiotics in animal husbandry (de Neeling *et al*., 2007). A large number of antimicrobials have been incorporated in the animal diets for prevention and growth promotion, which has frequently exposed animals to subtherapeutic concentrations of antibiotics (Dupont and Steele, 1987; Franco *et al*., 1990). Therefore, genes related to antimicrobial resistance may be transferred to bacteria in humans, which represent a potential risk for decreasing the efficacy of antibiotics used in human health (Smith *et al*., 2002).

Contaminated meat with AR enteric pathogens has been reported, including: *Salmonella*, *Campylobacter*, *Enterococcus*, and *Escherichia coli* (FDA, 2010). In addition, methicillin-resistant (MRSA) and MDR *Staphylococcus aureus* have been found in animals and meat (de Neeling *et al*., 2007; Waters *et al*., 2011; Buyukcangaz et al., 2013). However, there is no sufficient information about the prevalence of MRSA and MDR *S. aureus* strains in food of animal origin, and the route of transmission to animals and humans.

In humans, *S. aureus* can cause a wide variety of diseases, such as: food poisoning, pneumonia, wound, and nosocomial infections (Tiemersma *et al*., 2004; Kennedy *et al*., 2008). This opportunistic pathogen can be transmitted by the direct contact among animals and humans with an infectious disease. Moreover, the transmission may occur among people or animals that are colonized by *S. aureus* and are asymptomatic carriers (CFSPH, 2011).

Animals may be colonized by MRSA in their nares and skin (de Neeling *et al*., 2007; Moon *et al*., 2007; van Belkum *et al*., 2008; Persoons *et al*., 2009), increasing the risk of contamination of carcasses and meat during slaughtering (de Boer *et al*., 2009).

In the United States, the prevalence of *S. aureus* and MRSA nasal carriage in human is estimated at 29% and 1.5%, respectively (Gorwitz *et al*., 2008). Therefore, humans are also a potential source of contamination during processing and handling food. For this reason, undercooked food and food that do not need a further heat treatment to be consumed represent a higher risk to become vehicles in the spread of this pathogen (CFSPH, 2011).

In previous studies, the prevalence and genotyping of methicillin-susceptible *S. aureus* (MSSA) and MRSA strains isolated from animals and meat have been determined (Waters *et al*., 2011; de Neeling *et al*., 2007). Other past reports have characterized only MSSA and MRSA strains from humans (Tiemersma *et al*., 2004; Kennedy *et al*., 2008). Therefore, the source of emerging *S. aureus* strains that could cause infections and the genetic similarities between *S. aureus* strains isolated from different type of sources are not totally clear.

1.1. Characteristics of *Staphylococcus aureus*

Previously, the genus *Staphylococcus* had been classified within the *Micrococacaeae* Family. However, further studies of genetic homology demonstrated that the genera *Staphylococcus* and *Micrococcus* have an insufficient relationship. For that reason, *Staphylococcus* was included in the *Staphylococcaceae* Family, within the Order *Bacillales* (Euzéby, 1997). The name *Staphylococcus* comes from the Greek *staphyle*, meaning a bunch of grapes, due to the spherical shape (cocci with diameter between 0.5 and 1.5 µm) arranged in a grapelike clusters.

Staphylococci are Gram-positive, non-motile, non-spore forming, and facultative anaerobes. The main criterion to differentiate *Staphylococcus* from *Streptococcus* and *Enterococcus* is the synthesis of the enzyme catalase, which hydrolyzes hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). In addition, staphylococci exhibit a fermentative and oxidative metabolism of glucose, which can also be used to differentiate from micrococci (de Cueto and Pascual, 2009).

On non-selective media, *S. aureus* forms smooth and raised colonies, pigmented creamy yellow, due to the synthesis of a carotenoid pigment. This species has resistance to heat and drying, and exhibits halotolerance (7.5% of NaCl). On blood agar, most strains cause lysis of red blood cells mediated by β-hemolysin (β-hemolysis), resulting in a clear halo surrounding the colonies. *Staphylococcus aureus* can be differentiated from other species (with some exceptions) by the synthesis of coagulase, which enables the conversion of fibrinogen to fibrin, causing the clotting of plasma (Lowy, 1998; de Cueto and Pascual, 2009). In addition, *S. aureus* synthesizes a thermostable DNase which hydrolyses the phosphodiester bonds. This characteristic also allows for the identification of *S. aureus* (de Cueto and Pascual, 2009).

Staphylococcus aureus expresses many potential virulence factors. The microbial surface components recognizing adhesive matrix molecules (MSCRAMM) mediate the initial attachment to and invasion of host cells and tissues, evasion of immune responses and biofilm formation. The main MSCRAMM are: clumping factor, fibronectin-binding proteins, and bone sialoprotein-binding protein (Lowy, 1998; de Cueto and Pascual, 2009; Foster *et al*., 2014). In addition, *S. aureus* synthesizes a polysaccharide intercellular adhesin, which is involved in the biofilm formation that provides protection and resistance to cells within the biofilm (de Cueto and Pascual, 2009; Foster *et al*., 2014). The immune evasion is promoted by protein A, an extracellular adherence protein and cytotoxins (Panton-Valentine leukocidin [PVL], α-toxin). Moreover, the bacterium expresses enzymes that destroy tissues and facilitate the spread of the infection (lipases, hyaluronidases, and proteases). Other virulence factors are related to food poisoning and toxic shock syndrome: enterotoxins, toxic shock syndrome toxin 1, exfoliative toxins A and B, and α-toxin (Lowy, 1998; de Cueto and Pascual, 2009). All of these virulence factors in *S. aureus* promote the colonization and invasion, resulting in severe damage to the host.

1.2. Mechanisms of methicillin resistance

There are several antistaphylococcal agents, however, *S. aureus* has developed mechanisms to neutralize them. Therefore, MDR *S. aureus* strains have been found in a variety of sources and hosts (McDougal *et al*., 2003; Aydin *et al*., 2011; Waters *et al*., 2011).

In the early 1960s, MRSA associated with nosocomial infections emerged rapidly in Europe after the introduction of methicillin (the first semisynthetic penicillinase-resistant penicillins). At the end of the 1990s decade, community-associated MRSA (CA-MRSA) emerged worldwide (Lowy, 1998, 2003; Deurenberg and Stobberingh, 2008). Thus, the global spread of MRSA has increased the public health concern (Voss and Doebbling, 1995), due to the growing emergence of health care-associated (HA-MRSA) (Tiemersma *et al*., 2004), CA-MRSA (Kennedy *et al*., 2008) and livestock-associated (LA-MRSA) infections (Golding *et al*., 2010).

Methicillin is a β-lactam antibiotic, as well as penicillin G, oxacillin, ampicillin, amoxicillin and cephalosporins. In methicillin-susceptible *S. aureus* (MSSA), these antibiotics cause the inhibition of the last reaction of the cell wall synthesis called transpeptidation, where N-acetylmuramic acid is attached to the peptidoglycan. The transpeptidation is mediated by transpeptidases and carboxypeptidases called penicillin-binding proteins (PBPs) due to their affinity for penicillin. The β-lactam ring is covalently attached to a serine located in the active site of PBPs, causing the inactivation of transpeptidation and cell wall disruption. In addition, there are PBPs that inhibit autolytic cell-wall hydrolases, therefore, the attachment of penicillin to PBPs results in cell lysis (Marín and Gudiol, 2003; Romero, 2007).

One of the mechanisms of resistance to β-lactam antibiotics is the activity of the enzyme β-lactamase, which hydrolyzes the β-lactam ring. Penicillinase is a β-lactamase, which confers

resistance to penicillin and is encoded by the *blaZ* gene, located on a transposon within a plasmid with other genes associated with antimicrobial resistance (Lowy, 2003).

Methicillin-resistance in MRSA strains confers resistance to cephalosporins and penicillinase-resistant penicillins (Lowy, 1998). This is attributed to the low-affinity penicillin-binding protein 2a (PBP2a) (Hartman and Tomasz, 1981; Lim and Strynadka, 2002). Unlike the other PBPs, the PBP2a has an active site in which β-lactam antibiotics cannot bind. Therefore, the transpeptidation reaction can occur normally. Thus, the synthesis of the cell wall causes the survival of staphylococci that are exposed to high concentrations of these antimicrobials (Lim and Strynadka, 2002). The PBP2a is encoded by the *mecA* gene, located in a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Hartman and Tomasz, 1981). Transcriptional regulation of the *mecA* gene is accomplished by two proteins: MecI, repressor encoded by the *mecI* gene; and MecRI, signal transducer encoded by the *mecRI* gene. In the absence of β-lactam antibiotics, MecI binds the operator region, repressing the RNA transcription of both *mecA* and *mecI-mecRI* genes. Binding of β-lactam antibiotics to MecRI stimulates its autocatalytic activation. Active MecRI cleaves MecI into inactive fragments, allowing the transcription of both *mecA* and *mecI-mecRI* genes (Lowy, 2003).

In recent years, a novel *mecA* homolog gene ($mecA_{\mathrm{LGA251}}$ renamed as *mecC*) has been detected in *S. aureus* strains isolated from humans and animals, which exhibit methicillin resistance but test negative for the *mecA* gene. The *mecC* gene has 70% sequence homology to the *mecA* gene and is located on the staphylococcal cassette chromosome *mec* type XI (García-Álvarez *et al*., 2011; Ito *et al*., 2012; Laurent *et al*., 2012; Petersen *et al*., 2012).

1.3. Genotyping of MSSA and MRSA

Genotyping of *S. aureus* strains is not completely standarized, and different molecular methods have been used for many years (Tenover *et al*., 1994). Among the molecular methods to type MRSA strains are: pulsed-field gel electrophoresis (PFGE), that considers the macro-rectriction of genomic DNA; multilocus sequence typing (MLST), based on the allelic profile of seven housekeeping genes; and the *spa* typing, based on sequencing of the polymorphic X region of the *spa* gene that encodes the protein A in *S. aureus* (McDougal *et al*., 2003). For this reason, one strain can have multiple names (CFSPH, 2011). The Centers for Disease Control and Prevention (CDC) established a nomenclature system for *S. aureus* based on the PFGE patterns that were common in the US, listing eight original isolates, USA100 to USA800 (McDougal *et al*., 2003). The MLST types are named according to the sequence types (ST) with a number (e.g. ST398), while *spa* types are named with a 't' followed by a number (e.g. t011) (Cuny *et al*., 2010). Usually, PFGE and MLST classify isolates in similar clusters (Catry *et al*., 2010), which could contain different *spa* types. Therefore, one of the disadvantages of the *spa* typing is that the unrelated clonal lineages could have similar *spa* types (Van den Broek IV *et al*., 2009; Golding *et al*., 2008). This discrepancy with the results obtained by PFGE and MLST is due to the small fraction of the genome comprised by the *spa* typing (Golding *et al*., 2008). The PFGE method has proven to have a greater discriminatory power than MLST and *spa* typing methods. These techniques could be used to assess major changes in clonal lineages over time (McDougal *et al*., 2003). Therefore, a combination of two methods may be recommended in order to achieve a high accuracy in typing isolates (Tenover *et al*., 1994).

The PFGE technique has been used for differentiation between CA-MRSA (USA300 and USA400), and HA-MRSA strains (USA100 and USA200) (McDougal *et al*., 2003). Vandenesch

et al. (2003) determined that the CA-MRSA strains from three different continents harbored two genes: a type IV SCC*mec* cassette and the PVL locus, that carries the PVL-encoding gene. The PVL locus is carried on a bacteriophage that infects *S. aureus*, whereas the distribution of other toxin-encoding genes seems to be specific to the strains from each continent. Most CA-MRSA strains harbored the PVL locus (Baba *et al*., 2002; Dufour *et al*., 2002), which is a virulence factor related to severe skin infections, pneumonia, and tissue necrosis (Ebert *et al*., 2009).

Some sequence types related to HA-MRSA have been determined by MLST, such as: ST5, ST8, ST22, ST36, ST45, among others (Deurenberg *et al*., 2007), whereas ST30 and ST80 have been associated with CA-MRSA (Stenhem *et al*., 2010); and ST398 to LA-MRSA, particularly in pigs (Lewis *et al*., 2008; van Belkum *et al*., 2008; Krziwanek *et al*., 2009). Initially, the serotype ST398 was associated with pigs, however it has also been detected in humans, primarily pig farmers (van Belkum *et al*., 2008; Krziwanek *et al*., 2009; Pan *et al*., 2009; Golding *et al*., 2010). Moreover, MRSA ST398 has been recently associated with infections in humans that had contact with dairy cattle affected by sub-clinical mastitis (Soavi *et al*., 2010). Nevertheless, in Sweden two cases of ST398 t038 were reported in patients with no previous contact with animals (Welinder-Olsson *et al*., 2008). This suggests the spread and colonization of these strains, to people that are not involved in animal husbandry (Gibbs *et al*., 2006).

Methicillin-resistant *S. aureus* ST398 is non-typeable by PFGE since its DNA cannot be digested by the enzyme *Sma*I, due to the methylation of the *Sma*I recognition site caused by a methylation enzyme (Bens *et al*., 2006). Comparative assessment of PFGE fingerprints and *spa* types of MRSA ST398 strains obtained from patients in different countries have reported variations that suggest a molecular and geographic diversity (Golding *et al*., 2010). In addition, novel MRSA strains, such as ST9 t899 associated with pig farming, have also emerged

(Guardabassi *et al*., 2009). Therefore, the emergence of novel MRSA strains in swine, highlights the importance of creating strategies for permanent surveillance, and assessing the risk of transmission to humans related to pig farming.

1.4. Prevalence of MSSA and MRSA in animals, meat, and humans

The method used for isolation of MSSA and MRSA is not completely standarized. Therefore, the use of different methods may affect the results with regards to prevalence. Some analyzes have included solely plating using mannitol-salt agar (MSA) with 2 µg/mL oxacillin (Weese *et al*., 2006) or Baird Parker media (BP) (Aydin *et al*., 2011). Other studies, have used enrichment steps preceeding plating. Wertheim *et al*. (2001) developed a selective broth containing phenol red, mannitol, aztreonam, and ceftizoxime ($PHMB^+$), increasing the sensitivity of the detection of MRSA twofold. Broens *et al*. (2011) used a two-step enrichment protocol, Mueller-Hinton broth with 6.5% NaCl (MHB+6.5NaCl) and $PHMB^+$, followed by a chromogenic MRSA screening agar. Other authors have used: enrichment broth containing 7.5% NaCl, 1% mannitol and 2.5% yeast extract followed by a chromogenic medium (Zhang *et al*., 2011); trypticase soy broth supplemented with 10% NaCl and 1% sodium pyruvate followed by BP medium (Pu *et al*., 2009; Pu *et al*., 2011); $PHMB^+$ followed by plating on sheep blood agar and two selective agar media (Tenhagen *et al*., 2009). These methods suggest that adding an enrichment step followed by selective plating increases the sensitivity of detection.

Most animals can be colonized by *S. aureus* in the nares and skin (de Neeling *et al*., 2007; Moon *et al*., 2007; Lewis *et al*., 2008; van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009), therefore, there is a risk of contamination of carcasses and meat with MSSA and/or MRSA during slaughtering (de Boer *et al*., 2009). Recently, MRSA strains have been isolated from pigs, cows and chicken (de Neeling *et al*., 2007; Moon *et al*., 2007; Lewis *et*

al., 2008; van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009). In the Netherlands, de Neeling *et al*. (2007) have detected a high prevalence of MRSA ST398 (39%) in pigs and a high rate of resistance to different antibiotics (tetracycline, erythromycin, clindamycin, kanamycin, gentamicin, and tobramycin). In addition, they suggested the transmission of MRSA among animals within the pens in slaughterhouses. Guardabassi *et al*. (2009) found a prevalence of 16% in pigs in Hong Kong, which is lower than the prevalence reported in the former study. This could be due to a smaller sample size and the method of detection used. Since pigs have been found as a likely source of MRSA infections, it is necessary to study the epidemiology of this emerging zoonosis, determining the rate of transmission of MRSA from animals to humans, and person to person (Lewis *et al*., 2008).

In recent years, MRSA strains have been isolated from retail meat (pork, chicken, beef, turkey and lamb). Hanson *et al*. (2011) assessed different types of meat from supermarkets located in Iowa, USA. They detected two samples of pork meat contaminated with MRSA, with a prevalence around 1%. In Louisiana (USA), Pu *et al*. (2009) reported a prevalence of 45.6% of *S. aureus* in pork meat and 20% in beef, of which 5.6% and 3.3% were MRSA in pork meat and beef, respectively. A higher rate of MRSA, mainly ST398, has been found in retail meat in the Netherlands: 35% in turkey, 16% in chicken, 11% in pork, 10% in beef, and 6% in lamb (de Boer *et al*., 2009). Therefore, the latter results suggest that the method used could improve the level of detection of MRSA, which included two steps of selective enrichment followed by selective plating. Finally, the detection of MRSA in meat has increased the food safety concern regarding the meat production chain, resulting in the necessity of a surveillance system in coordination with different entities, such as: departments of health, laboratories of analysis, institutes of research, and food companies, among others.

In the United States, one in six people becomes sick as a result of foodborne illness each year, with a total of 48 million people. Among the main casual agents of foodborne diseases is *S. aureus*, with a total of 240,000 cases, corresponding to a gastrointestinal disease caused by enterotoxins (CDC, 2012). In contrast, MRSA are also associated with skin infections in the community, and severe cases are related to hospitalized patients affected by blood, surgical, or pneumonia infections (CDC, 2011). Nasal carriage of *S. aureus* and MRSA is approximately 29%, and 1.5% of the population in the United States, respectively (Gorwitz *et al*., 2008). A total of 478,000 cases of infections caused by *S. aureus* resulted in hospitalization in 2005, of which 50% were related to MRSA strains. Of 11,406 deaths associated with *S. aureus*, 6,639 cases were MRSA infections (Klein *et al*., 2007).

In North Dakota, statistics about the epidemiology of MRSA infections in humans are available. However, there is no information regarding to the prevalence of MRSA in animals and the contamination of meat. During 2011, a rate of 13 cases of MRSA infections per 100,000 people were reported. The rate of infections increased from 2000 to 2006, from 15 to 1,880 cases, respectively, decreasing markedly in 2007 (412 cases), with minor reductions in subsequent years (North Dakota Department of Health, Disease Control, 2011).

The infections caused by AR microorganisms have high direct and indirect costs. The average cost of hospitalization for MRSA infections is US$ 14,000 approximately, which is considerably high compared with US$ 7,600 for non-MRSA infections. Therefore, the total cost of hospitalization for MRSA infections in the United States is greater than 3 billion dollars annually (Elixhauser and Steiner, 2007). The total cost increases when indirect expenses are included, such as: sick leave, loss of earnings, disability, and mortality.

The control and prevention of MRSA infections in the United States is led by the CDC, which provides specific information and strategies for the treatment of MRSA infections. Decreasing MRSA infections in health care settings and in the community is a high priority to CDC. Thus, projects about the surveillance of MRSA infections have been developed with the collaboration of departments of health, hospitals, and medical centers, among others (CDC, 2011). However, more information about the genetic characteristics and similarities of MRSA strains and the route of transmission to animals and humans is needed. This could facilitate the development of the corrective actions to decrease the spread of MRSA infections.

1.5. Hypothesis and objectives

1.5.1. Hypothesis

Methicillin-susceptible (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains are present in meat-producing animals, retail raw meat, and humans, with a genetic relationship between them.

1.5.2. General objective

To determine the prevalence, molecular typing and the antimicrobial susceptibility of MSSA and MRSA in meat-producing animals, retail raw meat, deli meat, and humans, assessing the genetic relationship between isolates.

1.5.3. Specific objectives

- To determine the prevalence of *S. aureus* strains in animals, meat, deli meat, and humans.
- To determine the molecular characteristics and genotyping of MSSA and MRSA strains.
- To determine the antimicrobial resistance profiles of MSSA and MRSA strains.
- To compare a real time PCR assay with the culture method and conventional PCR technique, for detecting MSSA and MRSA in animals, meat, and deli meat.

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2. MOLECULAR TYPING OF *STAPHYLOCOCCUS AUREUS* AND METHICILLIN-RESISTANT *S. AUREUS* (MRSA) ISOLATED FROM ANIMALS AND RETAIL MEAT IN NORTH DAKOTA, UNITED STATES

2.1. Abstract

Several studies have reported the presence of *S. aureus* and MRSA in food animals and meat, suggesting the potential risk for humans. The objective of this study was to determine the prevalence and molecular typing of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in food-producing animals and retail meat in Fargo, North Dakota. A two-step enrichment followed by culture methods were used to isolate *S. aureus* from 167 nasal swabs from animals, 145 samples of retail raw meat, and 46 samples of deli meat. Positive isolates were subjected to multiplex polymerase chain reaction in order to identify the genes 16S rRNA, *mecA*, and Panton-Valentine Leukocidin. Pulsed-field gel electrophoresis and multilocus sequence typing were used for molecular typing of *S. aureus* strains. Antimicrobial susceptibility testing was carried out using the broth microdilution method. The overall prevalence of *S. aureus* was 37.2% (n=133), with 34.7% (n=58) of the animals positive for the organism. The highest prevalence was observed in pigs (50.0%) and sheep (40.6%) (p<0.05). Also, 47.6% (n=69) of raw meat samples were positive, with the highest prevalence in chicken (67.6%) and pork (49.3%) (p<0.05); and 13.0% (n=6) of deli meat was positive. Five pork samples (7.0%) were positive for MRSA, of which three were ST398 and two were ST5. All exhibited penicillin resistance and four were multidrug-resistant (MDR). The Panton-Valentine Leukocidin gene was not detected in any sample by multiplex polymerase chain reaction. The most common clones in sheep were ST398 and ST133, in pigs and pork both ST398 and ST9, and in chicken ST5. Most antimicrobial-susceptible *S. aureus* strains were ST5 isolated from

chicken. The MDR isolates were found in pigs, pork meat, and sheep. The presence of MRSA, MDR, and the subtype ST398 in the meat production chain and the genetic similarity between strains from pork meat and pigs suggest the possible contamination of meat during slaughtering and its potential transmission to humans.

2.2. Introduction

Outbreaks caused by antimicrobial-resistant (AR) bacteria is an established problem worldwide (DeWaal *et al*., 2011). One of these AR pathogens is methicillin-resistant *Staphylococcus aureus* (MRSA), which causes health care-associated MRSA (HA-MRSA) (Tiemersma *et al*., 2004), community-associated (CA-MRSA) (Kennedy *et al*., 2008), and livestock-associated (LA-MRSA) MRSA infections (Golding *et al*., 2010).

Most animals can become colonized with *S. aureus* (de Neeling *et al*., 2007; Moon *et al*., 2007; Lewis *et al*., 2008; van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009), and contamination of carcasses may occur during slaughtering (de Boer *et al*., 2009). Recently, MRSA strains have been isolated from several food-producing animals (de Neeling *et al*., 2007; Moon *et al*., 2007; Lewis *et al*., 2008; van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009); and from retail meat worldwide (de Boer *et al*., 2009; Pu *et al*., 2009; Lim *et al*., 2010; Weese *et al*., 2010; Bhargava *et al*., 2011; Hanson *et al*., 2011), representing a potential risk for its transmission to humans.

Methicillin resistance is attributed to the altered penicillin binding protein (PBP2a), encoded in the *mecA* gene, which has a reduced affinity for β-lactam antibiotics (Hartman and Tomasz, 1981; Van De Griend *et al*., 2009). The CA-MRSA strains are more likely to encode a virulence factor called Panton-Valentine leukocidin (PVL) toxin (Baba *et al*., 2002; Dufour *et al*., 2002),

associated with skin infections and tissue necrosis (Ebert *et al*., 2009). Therefore, the PVL toxin has been identified as a genetic marker for CA-MRSA strains (Vandenesch *et al*., 2003).

Different molecular techniques have been used for typing MRSA strains, such as pulsed-field gel electrophoresis (PFGE) based on macrorestriction patterns of genomic DNA; multilocus sequence typing (MLST) that determines the allelic profile of seven housekeeping genes; and *spa* typing based on the sequencing of the polymorphic X region of the protein A gene. It has been demonstrated that the discriminatory power of PFGE is greater than MLST and *spa* typing (McDougal *et al*., 2003; Malachowa *et al*., 2005). Tenover *et al*. (1994) suggest that a combination of two methods may provide more precision in epidemiological studies.

It has been demonstrated that MRSA strains causing CA-MRSA infections (USA300 and USA400) are different from those causing HA-MRSA infections (USA100 and USA200) (McDougal *et al*., 2003). The sequence type ST398 has been associated with livestock-associated MRSA (LA-MRSA) (Lewis *et al*., 2008; van Belkum *et al*., 2008; Welinder-Olsson *et al*., 2008; Krziwanek *et al*., 2009), however, the presence of ST398 and the emergence of infections in humans with livestock exposure, mostly pig farmers, has increased the public health concern (van Belkum *et al*., 2008; Krziwanek *et al*., 2009; Pan *et al*., 2009; Golding *et al*., 2010).

The aim of this study was to determine the prevalence, molecular typing, and genetic similarity of *S. aureus* and MRSA isolated from animals and retail meat in Fargo, ND.

2.3. Materials and methods

2.3.1. Samples

A total of 167 nasal swabs (sheep, *n*=64; pigs, *n*=60; cows, *n*=43) were collected from food-producing animals immediately after stunning at the Meat Lab (Department of Animal Sciences). Of these samples a total of 57 (sheep, *n*=14; pigs, *n*=18; cows, *n*=25) were obtained from sick

animals at the Veterinary Diagnostic Lab (North Dakota State University). Moreover, 145 raw meat (pork, *n*=71; chicken, *n*=37; beef, *n*=37) and 46 deli meat (ham, *n*=21; turkey, *n*=16; chicken, *n*=9) samples were randomly purchased from four supermarket chains in Fargo, ND.

Samples were collected between May 2010 and April 2011, immediately stored at 4°C, and processed within 6 h of collection.

2.3.2. Isolation of *S. aureus* and MRSA

The isolation was carried out by enrichment (de Boer *et al*., 2009) followed by plating steps on selective agar. Briefly, for the primary enrichment, 25 g of meat and 225 mL of Mueller-Hinton broth (Becton, Dickinson and Company [BD], Sparks, MD) with 6.5% sodium chloride (VWR International, West Chester, PA) (MHB+6.5% NaCl) were placed in a sterile stomacher bag and homogenized using a stomacher®400 circulator (Seaward, England) at 230 rpm for 90 s. The suspension was incubated for 18 to 20 h at 37°C. One milliliter of primary enrichment was inoculated into 9 mL of phenol red mannitol broth (BD) containing ceftizoxime (5 μg/mL, US Pharmacopeia, Rockville, MD) and aztreonam (75 μg/mL, Sigma Chemical Co., St. Louis, MO) ($PHMB^+$) (Wertheim *et al*., 2001), followed by incubation for 18 to 20 h at 37°C.

Nasal swabs were placed directly in 9 mL MHB+6.5% NaCl and incubated for 18 to 20 h at 37°C. Then, the procedure described above was carried out.

A loopful of secondary enrichment was struck directly to Baird-Parker medium with egg yolk tellurite supplement (BP) (according to manufacturer's recommendations) (BD) and incubated for 48 h at 37°C. Two presumptive *S. aureus* colonies on BP (black colonies surrounded by 2- to 5-mm clear zones) were transferred to Trypticase soy agar with 5% sheep blood (TSAII 5% SB) (BD) and incubated for 18 to 20 h at 37°C. Presumptive *S. aureus* on TSAII 5% SB (presence of β-hemolysis) was confirmed using Sensititre Gram Positive ID

(GPID) plates (Sensititre®, TREK Diagnostic Systems Ltd., Cleveland, OH). Confirmed colonies were stored frozen at -80°C in brain-heart infusion broth (BD) containing 20% glycerol until use.

2.3.3. Multiplex polymerase chain reaction (PCR)

All *S. aureus* strains were recovered from frozen stock to TSA plates and incubated at 37°C for 18 to 24 h. DNA extraction was carried out by suspending one colony in 50 µL of DNase/RNase-free distilled water, heating the suspension (99°C, 10 min) and then centrifugation (30,000 × *g*, 1 min) to remove cellular debris. The remaining DNA was transferred to a new tube and stored frozen at - 20°C until use.

Multiplex PCR assay for detection of 16S rRNA, *mecA* and PVL genes included 2 µL of the DNA template (described above) added to a 50 µL final reaction mixture: 1X Go Taq® Reaction Buffer (Promega, Madison, WI), 0.025 U/µL of Go Taq® DNA polymerase (Promega), 200 µM dNTP (Promega), and 1 µM of primers (16S rRNA, *mecA*, LukS/F-PV, Table 2.1) (Integrated DNA Technologies, Inc., Coralville, IA) (McClure *et al*., 2006).

Multiplex PCR settings were carried out according to Makgotlho *et al*. (2009), using a thermocycler (Eppendorf, Hamburg, Germany).

Ten microliters of the PCR amplicons were loaded into a 1.5% (wt/vol) agarose gel (Agarose ITM, Amresco, Solon, OH) in 1X TAE buffer using EzVision One loading dye (Amresco), and run at 100V in 1X TAE buffer for 1 h. A molecular weight marker 100-bp ladder (Promega) and a positive control (ATCC 33591) were included on each gel. Bands were visualized using an Alpha Innotech UV imager (FluorChem™).

Table 2.1. Nucleotide sequence of the primers used in multiplex polymerase chain reaction for detection of 16S rRNA, *mecA*, and Panton-Valentine leukocidin genes; and multilocus sequence typing analysis for detection of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* genes.

Primer	Oligonucleotide sequence	Amplicon Size (bp)
Staph 756 F	5'-AAC TCT GTT ATT AGG GAA GAA CA-3'	756
Staph 750 R	5'-CCA CCT TCC TCC GGT TTG TCA CC-3'	
mecA 1 F	5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3'	310
mecA-2 R	5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3'	
luk-PV-1 F	5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3'	433
luk-PV-2 R	5'-GCA TCA AGT GTA TTG GAT AGC AAA AGC-3'	
arcC F	5'-TTG ATT CAC CAG CGC GTA TTG TC-3'	456
arcC R	5'-AGG TAT CTG CTT CAA TCA GCG-3'	
aroE F	5'-ATC GGA AAT CCT ATT TCA CAT TC-3'	456
aroE R	5'-GGT GTT GTA TTA ATA ACG ATA TC-3'	
glpF F	5'-CTA GGA ACT GCA ATC TTA ATC C-3'	465
glpF R	5'-TGG TAA AAT CGC ATG TCC AAT TC-3'	
gmK F	5'-ATC GTT TTA TCG GGA CCA TC-3'	429
gmK R	5'-TCA TTA ACT ACA ACG TAA TCG TA-3'	
pta F	5'-GTT AAA ATC GTA TTA CCT GAA GG-3'	474
pta R	5'-GAC CCT TTT GTT GAA AAG CTT AA-3'	
tpi F	5'-TCG TTC ATT CTG AAC GTC GTG AA-3'	402
tpi R	5'-TTT GCA CCT TCT AAC AAT TGT AC-3'	
yqiL F	5'-CAG CAT ACA GGA CAC CTA TTG GC-3'	516
yqiL R	5'-CGT TGA GGA ATC GAT ACT GGA AC-3'	

16S rRNA, *mecA*, and Panton-Valentine leukocidin genes (McClure *et al.*, 2006).
arcC, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* genes (Enright *et al.*, 2000).

2.3.4. PFGE

The PulseNet protocol with slight modifications was used (McDougal *et al.*, 2003). Briefly, frozen isolates were struck in TSA plates and incubated at 37°C for 18 to 24 h. A single colony was inoculated into a second TSA plate and incubated at 37°C for 18 to 24 h. Colonies were

transferred to 5-mL polystyrene round-bottom tubes containing 2 mL of cell suspension buffer (100 mM Tris HCl [pH 8.0], Invitrogen; and 100mM EDTA [pH 8.0],Gibco), adjusting the concentrations to an absorbance of 0.9 to 1.1 in a spectrophotometer (Smart Spec^{TM} plus, Bio-Rad Laboratories, USA) at 610 nm. After that, the preparation, lysis, and washes of plugs, and then the *Sma*I enzyme restriction digestion were performed according to the PulseNet protocol. *Salmonella* Branderup H9812 was used as a DNA marker (Ribot *et al*., 2006).

The electrophoresis was carried out in a Chef Mapper (Bio-Rad Laboratories) PFGE rig, with initial switch time of 5 s, final switch time of 40 s, and total running time of 17 h 45 min.

After staining the gels with ethidium bromide (1.5 µg/mL), they were visualized using a UVP imager (UVP, Upland, CA). Macrorestriction patterns were compared using the BioNumerics Fingerprinting software (Ver 6.5 Applied Math, Austin, TX). The similarity index was calculated using the Dice coefficient, a band position tolerance of 1%, and an optimization of 0.5%. The unweighted-pair group method with arithmetic averages was used to construct a dendrogram, and clusters were selected using a cutoff at 80% (McDougal *et al*., 2003).

2.3.5. Multilocus sequence typing (MLST)

Briefly, *S. aureus* isolates were struck to TSA plates and incubated at 37°C for 18 to 24 h. Colonies were picked to 40 µL of single cell lysing buffer (50 µg/mL of Proteinase K, Amresco; in TE buffer [pH= 8]), and then lysed by heating to 80°C for 10 min followed by 55°C for 10 min in a thermocycler. The final suspension was diluted 1:2 in sterile water, centrifuged to remove cellular debris, and transferred to a sterile tube (Marmur, 1961).

The housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*, were amplified (Table 2.1) (Enright *et al*., 2000). All PCR reactions were carried out in 50-µL volumes: 1 µL of DNA template, Taq DNA polymerase (Promega) (1.25 U), 1X PCR buffer (Promega), primers (0.1

μM) (Integrated DNA Technologies, Inc.), and dNTPs (200 μM) (Promega). The PCR settings were adjusted according to Enright *et al*. (2000) using a thermocycler (Eppendorf). Ten microliters of the PCR products were loaded into 1% agarose gels in 1X TAE with EzVision One loading dye, and run at 100V in 1X TAE for 1 h. Images were captured using an Alpha Innotech imager.

After PCR, each amplicon was purified of amplification primer using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Purified DNA was sequenced at Iowa State University's DNA Facility (Ames, IA) using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence data were imported into DNAStar (Lasergene, Madison, WI), trimmed, and aligned to the control sequences (from the MLST site) and interrogated against the MLST database (http://saureus.mlst.net/). Sequence types were added to the strain information for analysis in BioNumerics software.

2.3.6. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) and the AR profiles of *S. aureus* isolates were determined using the broth microdilution method (CMV3AGPF, Sensititre®, Trek Diagnostics), according to the manufacturer's and the Clinical Laboratory Standards Institute guidelines (CLSI, 2009).

A total of 16 antimicrobials belonging to 13 classes were tested. Resistance to at least three classes of antibiotics was considered as multidrug-resistance (MDR) (Aydin *et al*., 2011).

2.3.7. Statistical analysis

Fisher's exact test was used to assess significance in prevalence of *S. aureus* and MRSA between animal and meat types (Moore et al., 2007). A significance level of $p<0.05$ and two-sided p-values were assessed using SAS software 9.2 (SAS Institute Inc., Cary, NC).

2.4. Results

Table 2.2 shows the prevalence of *S. aureus* in animals (34.7%, *n*=58), with a higher rate in swine and sheep ($p<0.05$); in raw meat (47.6%, *n*=69), with a higher rate in chicken and pork ($p<0.05$); and in deli meat (13.0%, *n*=6). The MRSA was detected in meat (five pork samples), representing a low prevalence ($p<0.05$). The PVL gene was not detected in any sample.

Table 2.2. Identification of 16S rRNA, *mecA* and Panton-Valentine leukocidin (PVL) genes in *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolates from animals and retail meat.

Source	No. of samples	Samples positive for *S. aureus*	Isolates with the specific gene		
			16S rRNA	*mecA*	PVL
Animal		----------No. (%)----------	--------------------No. (%)--------------------		
Sheep	71	26 (40.6)	26 (40.6)		
Pig	37	30 (50.0)	30 (50.0)		
Cow	37	2 (4.7)	2 (4.7)		
Total	145	58 (34.7)	58 (34.7)	0 (0.0)	0 (0.0)
Raw meat					
Pork	21	35 (49.3)	35 (49.3)	5 (7.0)	
Chicken	16	25 (67.6)	25 (67.6)		
Beef	9	9 (24.3)	9 (24.3)		
Total	46	69 (47.6)	69 (47.6)	5 (3.4)	0 (0.0)
Deli meat					
Ham		4 (19.0)	4 (19.0)		
Turkey		0 (0.0)	0 (0.0)		
Chicken		2 (22.2)	2 (22.2)		
Total		6 (13.0)	6 (13.0)	0 (0.0)	0 (0.0)

Most of the *Staphylococcus aureus* isolates from animals were resistant to penicillin, tetracycline, and lincomycin; and from raw meat to those antibiotics and erythromycin. All MRSA strains were resistant to penicillin, and most of them showed resistance to erythromycin, tetracycline, and lincomycin (Table 2.3).

A total of 47.7% (*n*=41) of the penicillin-resistant *Staphylococcus aureus* strains exhibited MICs between 0.5 and 1 µg/mL. However, MRSA strains had higher MICs for penicillin (1 - >16 µg/mL) (Table 2.4).

The rate of MDR strains was 41.4% (*n*=55); in animals was 51.7% (*n*=30), and in meat was 36.2% (*n*=25). Among MRSA strains, only one was not MDR, and the rest showed MDR to four classes of antimicrobials (Table 2.5).

Figure 2.1 shows a dendrogram displaying the macrorestriction patterns of *S. aureus* strains and the sequence types (STs). The largest cluster (cluster 4) contained *S. aureus* of porcine origin (animals and meat), all of which were ST9. *Staphylococcus aureus* isolates included in the second largest cluster (cluster 3) were obtained from poultry meat, and all but one was ST5. Two MRSA isolates were clustered in cluster 5, all from pork and ST5. The rest of the MRSA isolates were ST398 (not included in the dendrogram). A total of 34 *S. aureus* isolates (25.6%) were not included in the dendrogram because they could not be restricted with *Sma*I or *Xma*I during PFGE analysis and were ST398, isolated from sheep, pigs, and pork meat (data not shown).

2.5. Discussion

Both methods used for the confirmation of *S. aureus*, Sensititre identification plates and detection of the 16S rRNA gene by multiplex PCR, agreed with the results (Table 2.2). These results confirmed that the isolation method of two enrichment steps preceding plating is an appropriate method for recovering both *S. aureus* and MRSA from meat and animals. de Boer *et al*. (2009) used the same two-step enrichment, reporting a higher detection rate of MRSA.

Table 2.3. Antimicrobial resistance of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from animals and retail meat.

Antimicrobial		Animal (*n*=58)	Raw meat (*n*=69)	Deli meat (*n*=6)	Pork meat (*n*=5)
Subclass	Agent	Resistant *S. aureus* isolates			MRSA isolates
		No. (%)			No. (%)
Macrolides	Erythromycin	3 (5.2)	28 (40.6)	1 (16.7)	4 (80.0)
Tetracyclines	Tetracycline	47 (81.0)	29 (42.0)		4 (80.0)
Fluoroquinolones	Ciprofloxacin		2 (2.9)		
Phenicols	Chloramphenicol	3 (5.2)	2 (2.9)		
Penicillins	Penicillin	49 (84.5)	35 (50.7)	2 (33.3)	5 (100.0)
Aminoglycosides	Gentamicin	1 (1.7)	1 (1.4)		
	Kanamycin		2 (2.9)		1 (20.0)
	Streptomycin	6 (10.3)			
Streptogramin	Quinupristin/dalfopristin		2 (2.9)		
Lincosamides	Lincomycin	38 (65.5)	29 (42.0)	1 (16.7)	4 (80.0)

The following antimicrobials were tested using the National Antimicrobial Resistance Monitoring System (NARMS) panel: tigecycline (range 0.015-0.5 µg/mL); tetracycline (1-32); chloramphenicol (2-32); daptomycin (0.25-16); streptomycin (512-2048); tylosin tartrate (0.25-32); quinupristin/dalfopristin (0.5-32); linezoid (0.5-8); nitrofurantoin (2-64); penicillin (0.25-16); kanamycın (128-1024); erythromycin (0.25-8); ciprofloxacin (0.12-4); vancomycin (0.25-32); lincomycin (1-8); and gentamicin (128-1024). All isolates were susceptible to vancomycin, daptomycin, nitrofurantoin and linezolid.

Table 2.4. Minimum inhibitory concentrations (MICs) of resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* isolates from animals and retail meat.

Antimicrobial Agent (breakpoints)	Resistant *S. aureus* isolates	MIC (μg/mL)											
		0.5 - 1	2	4	>4	8	>8	16	>16	32	>32	256	>256
	----No.----	----------No. (%)----------											
Erythromycin (≥8 μg/mL)[a]	32						32 (100.0)						
Tetracycline (≥16 μg/mL)[a]	76							11 (14.5)		15 (19.7)	50 (65.8)		
Ciprofloxacin (≥4 μg/mL)[a]	2				2 (100.0)								
Chloramphenicol (≥32 μg/mL)[a]	5									3 (60.0)	2 (40.0)		
Penicillin (≥0.25 μg/mL)[a]	86	41 (47.7)	9 (10.5)	14 (16.3)		10 (11.6)		7 (8.1)	5 (5.8)				
Gentamicin (≥16 μg/mL)[a]	2											2 (100.0)	
Kanamycin (≥64 μg/mL)[a]	2												2 (100.0)
Streptomycin (≥8 μg/mL)[b]	6												6 (100.0)
Quinupristin/dalfopristin (≥8 μg/mL)[a]	2			1 (50.0)		1 (50.0)							
Lincomycin (≥4 μg/mL)[c]	68			4 (5.6)		3 (4.4)	61 (89.7)						

[a]Levels of MIC values against tested antibiotics (CLSI, 2009). [b]Levels of MIC values against tested antibiotics (Jarløv *et al*., 1997). [c]Levels of MIC values against tested antibiotics (Nemati *et al*., 2008).

Table 2.5. Antimicrobial resistance profiles of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from animals and retail meat.

Antimicrobial resistance profile	No. of antimicrobial subclasses resistant to	Animal (*n*=58)	Raw meat (*n*=69)	Deli meat (*n*=6)	Raw meat (*n*=5)
		S. aureus isolates with the specific profile			MRSA isolates with the specific profile
		-----------------No. (%)-----------------			------No. (%)------
ERY-PEN-TET-LINC-CHL-GEN-CIP-QUI	8		1 (1.4)		
ERY-PEN-TET-LINC-CHL-CIP-QUI	7		1 (1.4)		
ERY-PEN-TET-LINC-CHL-STR	6	2 (3.4)			
ERY-PEN-TET-LINC-KAN	5		1 (1.4)		
PEN-TET-LINC-CHL-STR	5	1 (1.7)			
PEN-TET-LINC-GEN	4	1 (1.7)			
PEN-TET-LINC-KAN	4		1 (1.4)		1 (20.0)
PEN-TET-LINC-STR	4	2 (3.4)			
ERY-PEN-TET-LINC	4	1 (1.7)	13 (18.8)		3 (60.0)
PEN-TET-LINC	3	22 (37.9)	1 (1.4)		
PEN-LINC-STR	3	1 (1.7)			
ERY-PEN-LINC	3		2 (2.9)		
ERY-TET-LINC	3		5 (7.2)		

Ciprofloxacin (CIP); chloramphenicol (CHL); erythromycin (ERY); gentamicin (GEN); kanamycin (KAN); lincomycin (LINC); quinupristin/dalfopristin (QUI); penicillin (PEN); streptomycin (STR); and tetracycline (TET).

Table 2.5. Antimicrobial resistance profiles of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from animals and retail meat (continued).

Antimicrobial resistance profile	No. of antimicrobial subclasses resistant to	Animal (*n*=58)	Raw meat (*n*=69)	Deli meat (*n*=6)	Raw meat (*n*=5)
		S. aureus isolates with the specific profile			MRSA isolates with the specific profile
		-----------------No. (%)-----------------			------No. (%)------
PEN-LINC	2	4 (6.9)	1 (1.4)	1 (16.7)	
PEN-TET	2	12 (20.7)	2 (2.9)		
TET-LINC	2	3 (5.2)			
ERY-LINC	2		3 (4.3)		
ERY-PEN	2		2 (2.9)		1 (20.0)
LINC	1	1 (1.7)			
PEN	1	3 (5.2)	10 (14.5)	1 (16.7)	
TET	1	3 (5.2)	4 (5.8)		
ERY	1			1 (16.7)	
Susceptible to all tested	0	2 (3.4)	22 (31.9)	3 (50.0)	

Ciprofloxacin (CIP); chloramphenicol (CHL); erythromycin (ERY); gentamicin (GEN); kanamycin (KAN); lincomycin (LINC); quinupristin/dalfopristin (QUI); penicillin (PEN); streptomycin (STR); and tetracycline (TET).

It is well known that animals are natural reservoirs of *S. aureus*; in this study, positive nasal swabs were obtained from sheep, pigs, and cows. Other studies have detected a higher prevalence of *S. aureus* in sheep (57%) and cow (14%) (Mørk *et al*., 2012); however, the prevalence in pigs has been reported to vary widely (6-57%) (Khalid *et al*., 2009; Lowe *et al*., 2011). The recovery of *S. aureus* in meat in our study was higher than previous studies (39.2% and 14.4%) (Pu *et al*., 2009; Aydin *et al*., 2011). The prevalence of *S. aureus* in ham was 19%, which was considerably lower than the prevalence reported by Atanassova *et al*. (2001). There is limited information about the prevalence of *S. aureus* and MRSA in processed retail meat products, and this study provides some information as to the potential exposure of consumers through consumption of deli meat that typically do not need heating prior to consumption.

In this study, MRSA was not detected in animals; however, a prevalence of MRSA in swine ranging from 10% to 71% has been detected previously (Köck *et al*., 2009; Smith *et al*., 2009; Tenhagen *et al*., 2009). The low rate of MRSA in pork raw meat (3.4%) determined in this study agreed with the low prevalence reported by other authors (de Boer *et al*., 2009; Pu *et al*., 2009).

Most of the *S. aureus* strains isolated from animals exhibited resistance to the same antimicrobials reported by other authors (Nemati *et al*., 2008; Huber *et al*., 2010) (Table 2.3). The AR bacteria in animals have increased over time due to the frequent use of antimicrobial agents at the farm level (de Neeling *et al*., 2007; Nemati *et al*., 2008). Therefore, controlling the use of antibiotics in farming could limit the risk of transmission of AR pathogens among animals and potentially to humans (Huber *et al*., 2010).

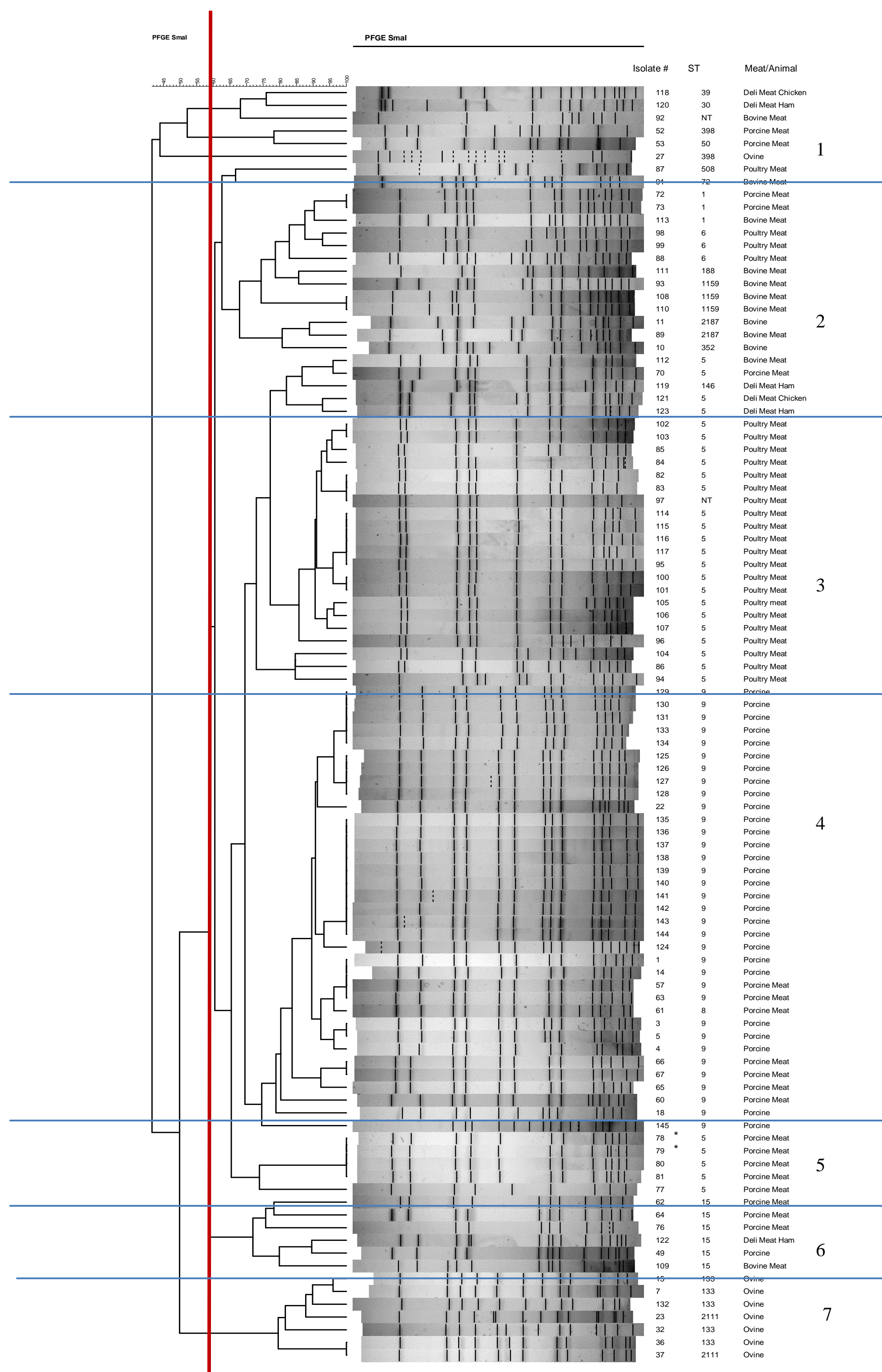

Figure 2.1. Dendrogram showing the genetic similarity of 100 *S. aureus* isolates. The scale indicates levels of similarity, numbers represent the samples codes, followed on the right by the sequence type (ST) and the type of the sample. **mecA*-positive *S. aureus* strains in pork meat.

Other authors have also determined a higher occurrence of resistance to penicillin, tetracycline, and erythromycin in *S. aureus* strains isolated from retail meat and different food samples (Aydin *et al*., 2011; Pu *et al*., 2011). Penicillin resistance has been reported to spread rapidly among *S. aureus* strains being facilitated by plasmids and is the most frequently reported resistance detected in foodborne *S. aureus* (Aydin *et al*., 2011).

Antimicrobial-resistant *S. aureus* exhibited a MIC for erythromycin and lincomycin (>8 µg/mL) lower than the MIC determined by Nemati *et al*. (2008). The MIC of tetracycline (>32 µg/mL) and penicillin (0.5-1 µg/mL) concurred with the results reported by Nemati *et al*. (2008).

All *S. aureus* isolates examined in this study were susceptible to daptomycin, linezolid, nitrofurantoin and vancomycin, concurring with the results reported by Pu *et al*. (2011).
The clustering of isolates obtained by PFGE agreed well with the MLST types (i.e., the identical restriction patterns or patterns that differed at two to six bands had an identical ST) (Fig. 1). Restriction patterns with the same numbers of bands represent the same strain; patterns that differ up to three fragments represent strains that are closely related; and isolates that differs at four to six bands may have the same genetic lineage (Tenover *et al*., 1995).

The major clones identified corresponded to ST9 and ST5. The emergence of ST9 in pigs was first reported in 2008 by Guardabassi *et al*. (2009) in Hong Kong, disseminating later as demonstrated in this study. The genetic relatedness between *S. aureus* strains ST9 from pigs and pork meat may suggest the possible contamination of meat during slaughtering. Previously, ST5 was associated with poultry (Hasman *et al*., 2010) and poultry meat (Waters *et al*., 2011). In this study, the majority of strains isolated from chicken were ST5, which can also suggest the contamination of meat during slaughtering. A high prevalence of MSSA ST398 strains was

found, which may indicate the potential risk for humans to acquire this emerging sequence type that has potential for causing infection.

The MRSA isolates had the same MLST allelic profile and indistinguishable PFGE patterns than two methicillin-susceptible *S. aureus* (MSSA) strains, all obtained from pork. The close genetic similarity of the MRSA and MSSA isolates may be due to the acquisition of the *mecA* gene by horizontal transfer of SCC*mec* from MRSA strains to MSSA lineages (Enright *et al*., 2000; Wielders *et al*., 2001; de Neeling *et al*., 2007; Guardabassi *et al*., 2009).

Most of the *S. aureus* isolates susceptible to all antimicrobial agents were obtained from chicken, of which 76% were ST5. Previously, other authors have reported MDR in *S. aureus* from food samples at a lower rate compared with this study (Aydin *et al*., 2011; Nam *et al*., 2011) (Table 2.4). Multidrug-resistant isolates from pork were mainly ST398 (60%) (not included in the dendrogram) and ST9 (30%). All MDR strains from sheep were ST398 (not included in the dendrogram). The multidrug resistance can be due to the presence of other antibiotic resistance genes, such as *dfrK* (resistance to trimethoprim) (Kadlec and Schwarz, 2009) and *cfr* (MDR gene) (Kehrenberg *et al*., 2009).

2.6. Conclusion

The genetic relationship between strains isolated from animals and meat, suggests the likely contamination of meat during slaughtering. Although the MRSA prevalence in raw meat is low, the prevalence of MDR *S. aureus* and ST398 is higher; therefore, the risk of transmission through the meat production chain cannot be ignored.

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3. MULTIPLEX REAL-TIME PCR FOR DETECTION OF *STAPHYLOCOCCUS AUREUS*, *MECA,* AND PANTON-VALENTINE LEUKOCIDIN (PVL) GENES FROM SELECTIVE ENRICHMENTS FROM ANIMALS AND RETAIL MEAT

3.1. Abstract

The need for rapid detection of MRSA has become an important goal in the microbiological analysis. The aim of this study was to compare a real-time PCR assay, with a conventional culture/PCR method, to detect *S. aureus*, *mecA* and Panton-Valentine Leukocidin (PVL) genes in animals and retail meat, using a two-step selective enrichment protocol. A total of 234 samples were examined (77 animal nasal swabs, 112 retail raw meat, and 45 deli meat). The multiplex real-time PCR targeted the genes: *nuc* (identification of *S. aureus*), *mecA* (associated with methicillin resistance), PVL (virulence factor), and the primary and secondary enrichment samples were assessed. The conventional culture/PCR method included the two-step selective enrichment, selective plating, biochemical testing, and multiplex PCR for confirmation. Of a total of 234 samples, the conventional culture/PCR method recovered 95 positive *S. aureus* samples. Application of real-time PCR on samples following primary and secondary enrichment detected *S. aureus* in 111 and 120 samples, respectively. For detection of *S. aureus*, the *k* statistic was 0.68 to 0.88 (from substantial to almost perfect agreement) and 0.29 to 0.77 (from fair to substantial agreement) for primary and secondary enrichments, respectively, using real-time PCR. For detection of *mecA* gene, the *kappa* statistic was 0 to 0.49 (from no agreement beyond that expected by chance to moderate agreement) for primary and secondary enrichment samples. Two pork samples were *mecA* gene positive by all methods. The real-time PCR assay detected the *mecA* gene in some samples that were negative for *S. aureus*, but positive for *Staphylococcus* spp. The PVL gene was not detected in any sample by the conventional

culture/PCR method or the real-time PCR assay. Among *S. aureus* isolated by conventional culture/PCR method, the sequence type ST398, and multidrug-resistant strains were found in animals and raw meat samples. The real-time PCR assay may be recommended as a rapid method for the detection of *S. aureus* and the *mecA* gene, with further confirmation of MRSA using the standard culture method.

3.2. Introduction

Staphylococcus aureus is an important cause of a wide variety of diseases in humans such as: food poisoning, pneumonia, wound, and nosocomial infections (Tiemersma *et al*., 2004; Kennedy *et al*., 2008). There are many anti-staphylococcal agents; however, the bacterium has developed mechanisms to neutralize them such as the methicillin resistance mechanism (Lowy, 2003). Methicillin-resistant *S. aureus* (MRSA) is an increasing cause of health care-associated (HA-MRSA) (Tiemersma *et al*., 2004), community-associated (CA-MRSA) (Kennedy *et al*., 2008), and livestock-associated (LA-MRSA) infections worldwide (Goldwing *et al*., 2010).

The altered penicillin-binding protein (PBP2a or PBP2') is associated with methicillin resistance. This protein has a reduced affinity for β-lactam antibiotics (Hartman and Tomasz, 1981; Van De Griend *et al*., 2009), and is encoded by the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) (Hartman and Tomasz, 1981). The CA-MRSA strains are more likely to encode the Panton–Valentine leukocidin (PVL) toxin, which is a pore-forming toxin considered as a virulence factor (Baba *et al*., 2002; Dufour *et al*., 2002). The PVL toxin has been related to life-threatening CA-MRSA infections and deaths, primarily severe skin infections and tissue necrosis (Ebert *et al*., 2009).

In the United States, approximately 29% (78.9 million people) and 1.5% (4.1 million) of the population were estimated to be nasal carriers of *S. aureus* and MRSA, respectively (Gorwitz *et*

al., 2008). An estimated 478,000 hospitalizations corresponded to *S. aureus* infections, of which 278,000 hospitalizations were attributed to MRSA infections in 2005 (Klein *et al*., 2007). In addition, the carriage of MRSA in meat-producing animals (van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009) and the contamination of meat with MRSA (de Boer *et al*., 2009; Buyukcangaz *et al*., 2013; O'Brien *et al*., 2012) have increased the concern that food may serve as a vehicle to transmit MRSA to the human population (O'Brien *et al*., 2012).

Different culture methods have been used to detect MRSA. Generally, conventional microbiological procedures are laborious, since they require the isolation of *S. aureus* before assessing methicillin resistance. However, culture methods are still considered as standard methods for traditional confirmation of *S. aureus*. Wertheim *et al*. (2001) developed a selective media containing phenol red, mannitol, and antibiotics (aztreonam and ceftizoxime), increasing the sensitivity of the detection of MRSA after 48 h of incubation, but at the expense of longer time needed for confirmation. The isolation and identification of MRSA, including selective enrichment and plating on selective agars, followed by confirmation using biochemical testing and/or PCR assays, requires 3 to 7 days approximately (de Boer *et al*., 2009; Buyukcangaz *et al*., 2013; Zhang *et al*., 2011). Therefore, development of a rapid method for detection of MRSA has become an important need in the microbiological analysis of samples especially those where there is a potential risk of exposure for humans.

Real-time PCR technology has been used as an alternative to culture methods for the rapid detection of *S. aureus* and MRSA. Real-time PCR may decrease the time of analysis to 18 h after consecutive broth enrichment in clinical samples (Söderquist *et al*., 2012); or <2 h in positive blood cultures (Thomas *et al*., 2007; Kilic *et al*., 2010). However, most studies have used real-time PCR to detect MRSA in clinical samples and isolates and a few studies have evaluated the

application of this method for the detection of MRSA in animals (Anderson and Weese, 2007; Morcillo *et al*., 2012) and meat (de Boer *et al*., 2009; Weese *et al*., 2010; Waters *et al*., 2010).

Since *S. aureus* and MRSA have been found in food-producing animals and retail meat, increasing the concern about the exposure for humans through the food chain, there is a need to decrease the time of analysis. We analyzed samples obtained from animals and retail meat using primary and secondary selective enrichments in order to detect *nuc* (identification of *S. aureus*), *mecA* (associated with methicillin resistance), and PVL (virulence factor) genes using a multiplex real-time PCR assay. The results were compared with the results from a culture method, considered as the standard method, which also included the two-step selective enrichment, followed by selective plating, biochemical testing, and conventional multiplex PCR. Positive samples obtained with the culture method were characterized by multilocus sequence typing (MLST) and the antimicrobial resistance profiles were obtained.

3.3. Materials and methods

3.3.1. Samples

A total of 77 nasal swabs (Becton, Dickinson and Company, Sparks, MD, USA) were collected from animals (sheep, *n*=35; pigs, *n*=28; cows, *n*=14) sampled immediately after stunning at the Meat Lab (Department of Animal Sciences); and at the Veterinary Diagnostic Lab at North Dakota State University, Fargo, ND. Animal samples were collected during the period May 2010 to April 2011. The protocol of sampling was approved by the North Dakota State University Institutional Biosafety Committee (B10014).

In addition, 112 retail raw meat (pork, *n*=39; chicken, *n*=37; beef, *n*=36) and 45 deli meat (ham, *n*=20; turkey, *n*=16; chicken, *n*=9) samples were randomly purchased from four different

supermarket chains in Fargo, ND. Sampling visits were made between June 2010 and January 2011. All samples were immediately stored at 4°C and processed within six hours of collection.

3.3.2. Culture method

Staphylococcus aureus were isolated by the two-step selective enrichment procedure according to the method described by de Boer *et al*. (2009) followed by plating steps on selective agar. Briefly, for the primary enrichment, a 25 g sample of retail meat and 225 mL of MHB+6.5%NaCl (Mueller-Hinton broth [Difco, Becton, Dickinson, Sparks, MD, USA] with added 6.5% sodium chloride [VWR International, West Chester, PA, USA]) were placed in a sterile stomacher bag and homogenized using a stomacher®400 circulator (Seaward, England) at 230 rpm for 90 seconds. The suspension was incubated for 18 to 20 h at 37ºC. Following primary enrichment, a secondary enrichment was used by inoculating 1 mL of the primary enrichment broth into 9 mL of PHMB$^+$ (D-mannitol in phenol red mannitol broth base [Difco, Becton, Dickinson, Sparks, MD, USA] containing ceftizoxime [5 µg mL^{-1}, US Pharmacopeia, Rockville, MD, USA] and aztreonam [75 µg mL^{-1}, Sigma Chemical CO., Louis, MO, USA] according to Wertheim *et al*. [2001]), followed by incubation for 18 to 20 h at 37ºC. Nasal swabs from animals were placed directly in 9 mL MHB+6.5%NaCl and incubated for 18 to 20 h at 37ºC. Then, the secondary enrichment was used following the procedure described above.

Following incubation of the secondary enrichment broth, all samples were struck directly to BP medium (Baird-Parker medium [Difco, Becton, Dickinson, Sparks, MD, USA]) supplemented with egg yolk tellurite according to manufacturer's recommendations and incubated for 48 h at 37°C. Presumptive *S. aureus* colonies (black colonies surrounded by 2 to 5 mm clear zones) were transferred to TSA II 5%SB plates (Trypticase soy agar with 5% sheep blood [Difco, Becton, Dickinson, Sparks, MD, USA]) and incubated for 18 to 20 h at 37ºC.

Presumptive *S. aureus* colonies (presence of β-haemolysis) were confirmed using Sensititre Gram Positive ID (GPID) plates (Sensititre®, TREK Diagnostic Systems Ltd., Cleveland, OH, USA) according to the manufacturer's instructions.

3.3.3. Conventional multiplex PCR method

Confirmed *S. aureus* strains were recovered from frozen stock to TSA plates (Trypticase soy agar [Difco, Becton, Dickinson, Sparks, MD, USA]) and incubated at 37°C for 18 to 24 h. DNA extraction was carried out by suspending one colony in 50 µL of DNase/RNase-free distilled water (Gibco Invitrogen, Grand Island, NY, USA), heating (99°C, 10 min) and centrifugation (30,000×*g*, 1 min) to remove cellular debris. The remaining DNA was transferred to a new tube and stored at -20°C until use.

A multiplex PCR assay for the detection of 16S rRNA (identification of *S. aureus*), *mecA* (associated with methicillin resistance) and PVL-encoding genes (virulence factor) (Table 3.1) included 2 µL of the DNA template (described above) added to a 50 µL final reaction mixture containing: 1X Go Taq® Reaction Buffer (pH 8.5), 0.025 U μL^{-1} of Go Taq® DNA polymerase, 200 µM dNTP (Promega, Madison, WI, USA) and 1 µM of primers (16S rRNA, *mecA*, LukS/F-PV) (Integrated DNA Technologies, Inc., Coralville, IA, USA).

Multiplex PCR reactions were carried out in a thermocycler (Eppendorf, Hamburg, Germany), and the PCR conditions were adjusted according to the protocol described by Makgotlho *et al*. (2009) as follows: initial denaturation at 94°C for 10 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 75 s followed by another 25 cycles of 94°C for 45 s, 50°C for 45 s and a final extension step at 72°C for 10 min. An external positive (DNA from MRSA ATCC 33591, positive for *mecA* and PVL genes) and negative control (DNase/RNase-free distilled water) were included with each run.

Table 3.1. Nucleotide sequence of the primers and probes used in conventional multiplex PCR and multiplex real-time PCR.

Primer or probe name	Sequence (5'→3')	5' Reporter dye 3' Quencher
16S rRNA†		
Staph-756F	AAC TCT GTT ATT AGG GAA GAA CA	
Staph-750R	CCA CCT TCC TCC GGT TTG TCA CC	
nuc‡		
nuc For	CAA AGC ATC AAA AAG GTG TAG AGA	
nuc Rev	TTC AAT TTT CTT TGC ATT TTC TAC CA	Texas Red
nuc Probe	TTT TCG TAA ATG CAC TTG CTT CAG GAC CA	Iowa Black
mecA		
mecA-1F†	GTA GAA ATG ACT GAA CGT CCG ATA A	
mecA-2F†	CCA ATT CCA CAT TGT TTC GGT CTA A	
mecA For‡	GGC AAT ATT ACC GCA CCT CA	
mecA Rev‡	GTC TGC CAC TTT CTC CTT GT	FAM†
mecA Probe‡	AGA TCT TAT GCA AAC TTA ATT GGC AAA TCC	TAMRA†
PVL		
luk-PV-1F†	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	
luk-PV-2R†	GCA TCA AGT GTA TTG GAT AGC AAA AGC	
PVL For‡	ACA CAC TAT GGC AAT AGT TAT TT	
PVL Rev‡	AAA GCA ATG CAA TTG ATG TA	Cy5†
PVL Probe‡	ATT TGT AAA CAG AAA TTA CAC AGT TAA ATA TGA	Iowa Black

†Conventional multiplex PCR, according to McClure *et al*. (2006).
‡Multiplex real-time PCR, according to McDonald *et al*. (2005).

Ten microliters of PCR amplicons were loaded into a 1.5% (wt/vol) agarose gel (Agarose I^{TM}) using EzVision One loading dye (Amresco, Solon, OH, USA) and electrophoresis was carried out in 1X TAE buffer at 100 v for 1 h. A molecular weight marker 100-bp ladder (Promega, Madison, WI, USA) were included on each gel. Bands were visualized using an Alpha Innotech UV imager (FluorChem^{TM}).

3.3.4. Multiplex real-time PCR assay

The DNA was extracted from the primary and secondary enrichment broths of the animal and meat samples using the boiling method described previously by De Medici *et al*. (2003). Five microliters of DNA template extracted was used in the real-time iQ^{TM} Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), in a final volume of 20 µL per reaction.

The real-time PCR assay targeted: *nuc* (identification of *S. aureus*), *mecA* (associated with methicillin resistance) and PVL-encoding genes (virulence factor) (Table 3.1).

The final concentrations in the reaction mixture were: 300 nM of primers (forward and reverse), 200 nM of fluorogenic probes (Applied Biosystems, Foster City, CA, USA), and 1X iQ^{TM} Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's recommendations.

The thermal cycling conditions were adjusted to an initial denaturation of 3 min at 95°C, followed by 40 PCR cycles of 95°C for 15 s and 55°C for 1 min, using an iCycler IQ^{TM} real time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). An external positive control (DNA from MRSA ATCC 35591, positive for *mecA* and PVL genes) and an external negative control (DNase/RNase-free distilled water) were included with each plate. Data analysis was carried out using the iCycler software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).

3.3.5. Characterization of *S. aureus* strains isolated by culture method

3.3.5.1. Multilocus sequence typing (MLST)

Briefly, *S. aureus* isolates were struck to TSA plates and incubated at 37°C for 18 to 24 h. Colonies were picked to 40 µL of single cell lysing buffer (50 µg/mL of Proteinase K, Amresco; in TE buffer [pH=8]), and then lysed by heating to 80°C for 10 min followed by 55°C for 10 min in a thermocycler. The final suspension was diluted 1:2 in sterile water, centrifuged to remove

cellular debris, and transferred to a sterile tube (Marmur, 1961). The housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*, were amplified (Enright *et al*., 2000). All PCR reactions were carried out in 50-µL volumes: 1 µL of DNA template, Taq DNA polymerase (Promega) (1.25 U), 1X PCR buffer (Promega), primers (0.1 µM) (Integrated DNA Technologies, Inc.), and dNTPs (200 µM) (Promega). The PCR settings were adjusted according to Enright *et al*. (2000) using a thermocycler (Eppendorf). Ten microliters of the PCR products were loaded into 1% agarose gels in 1X TAE with EzVision One loading dye, and run at 100V in 1X TAE for 1 h. Images were captured using an Alpha Innotech imager. After PCR, each amplicon was purified of amplification primer using the QIAquick®PCR Purification Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Purified DNA was sequenced at Iowa State University's DNA Facility (Ames, IA) using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence data were imported into DNAStar (Lasergene, Madison, WI), trimmed, and aligned to the control sequences (from the MLST site) and interrogated against the MLST database (http://saureus.mlst.net/). Sequence types were added to the strain information for analysis in BioNumerics software.

3.3.5.2. Resistance profiles

The antimicrobial resistance (AR) profiles of *S. aureus* isolates (*n*=95) were determined using the broth microdilution method (CMV3AGPF, Sensititre®, Trek Diagnostics), according to the manufacturer's and the National Antimicrobial Resistance Monitoring System (NARMS) guidelines for animal isolates (NARMS, 2012). Antimicrobials in the panel and their resistance breakpoints were as follows: erythromycin (≥8 µg/mL), tetracycline (≥16 µg/mL), ciprofloxacin (≥4 µg/mL), chloramphenicol (≥32 µg/mL), penicillin (≥16 µg/mL), daptomycin (no interpretative criteria), vancomycin (≥32 µg/mL), nitrofurantoin (≥128 µg/mL), gentamicin

(>500 µg/mL), quinupristin/dalfopristin (≥4 µg/mL), linezolid (≥8 µg/mL), kanamycin (≥1024 µg/mL), tylosin (≥32 µg/mL), tigecycline (no interpretative criteria), streptomycin (>1000 µg/mL), and lincomycin (≥8 µg/mL). Resistance to at least three classes of antibiotics was considered as multidrug resistance (MDR) (Aydin *et al*., 2011).

3.3.6. Statistical analysis

The 95% confidence intervals (CI) for prevalence were obtained, using the plus four estimate when positive or negative samples were less than 15. The Chi-square test was used to assess the significance in proportion of positive samples between sample types, only if no more than 20% of the expected counts were less than 5 and all individual expected counts were 1 or greater (Moore *et al*., 2007). On the contrary, Fisher's exact test was used with two-sided *p*-values. SAS software version 9.2 (SAS Institute Inc., Cary, NC) was used to assess significance with $p<0.05$.

As there is no true gold standard method for *S. aureus* and MRSA detection, the *k* statistic was calculated to compare agreement between real-time PCR assay (using primary and secondary enrichment) and conventional culture/PCR method.

3.4. Results

The culture method included a biochemical identification to confirm *S. aureus*, which agreed with the results of the conventional multiplex PCR that detected the gene 16S rRNA. This method detected 95 positive *S. aureus* samples from a total of 234 samples collected (Table 3.2). The multiplex real-time PCR assay using primary and a secondary enrichments, recovered *S. aureus* (detection of *nuc* gene) from 111 and 120 samples of 234 samples respectively.

By the conventional culture/PCR method alone, the rate of positive *S. aureus* samples was found to be 41.6% ($CI_{95\%}$, 30.6-52.6%) in animals and 51.8% ($CI_{95\%}$, 42.5-61.0%) in raw meat

samples, and a significantly lower rate of 11.1% ($CI_{95\%}$, 4.5-24.1%) was observed in deli meat ($p<0.05$). Using the primary enrichment samples and real-time PCR, a significantly higher recovery of *S. aureus* ($p<0.05$) was found in animals 55.8% ($CI_{95\%}$, 44.8-66.9%) and raw meat 57.1% ($CI_{95\%}$, 47.9-66.3%) than in deli meat samples 8.9% ($CI_{95\%}$, 3.1-21.4%). However, no significant difference ($p\geq0.05$) was found between the rate of positive *S. aureus* samples in animals (53.2%) ($CI_{95\%}$, 42.1-64.4%), raw meat (53.6%) ($CI_{95\%}$, 44.3-62.8%) and deli meat (42.2%) ($CI_{95\%}$, 27.8-56.7%), when the secondary enrichment samples were assessed by real-time PCR. A significantly higher recovery of *S. aureus* ($p<0.05$) was obtained from deli meat when the secondary enrichment samples were tested by real-time PCR.

The *mecA* gene was detected in two pork meat samples (5.4%) ($CI_{95\%}$, 0.7-18.8%) by the conventional multiplex PCR preceded by the culture method, and by assessing the primary and secondary enrichment samples by real-time PCR. The real-time PCR analysis detected the *mecA* gene using both enrichments in samples that were negative by conventional multiplex PCR in two pork meat and three deli meat samples. Using the primary enrichment, the real-time PCR detected the *mecA* gene in one sample isolated from a sheep, and one from pork meat, which were negative using the secondary enrichment. Using the secondary enrichment, the real-time PCR detected the *mecA* gene from one sample isolated from a pig, one from pork meat, and two from deli meat, which were negative using the primary enrichment. The PVL gene was not detected in any sample by the conventional culture/PCR method or the real-time PCR assay.

Table 3.3 shows the results of real-time PCR using primary and secondary enrichments on the detection of *S. aureus* compared with a conventional culture/PCR method. Total agreement and the *k* statistic for real-time PCR using the primary enrichment samples were 85.7% (k=0.72, $CI_{95\%}$, 0.62-0.82), 83.9% (k=0.68, $CI_{95\%}$, 0.59-0.76), and 97.8% (k=0.88, $CI_{95\%}$, 0.78-0.97) for

animals, raw meat, and deli meat respectively. For real-time PCR using the secondary enrichment samples, the total agreement and the *k* statistic were 88.3% (k=0.77, $CI_{95\%}$: 0.67-0.86), 87.5% (k=0.75, $CI_{95\%}$: 0.67-0.83), and 68.9% (k=0.29, $CI_{95\%}$: 0.16-0.43) for animals, raw meat, and deli meat, respectively. Positive agreement (sensitivity) was 100% for animal samples using both enrichments. For animals and raw meat, a higher negative agreement (specificity) was obtained for real-time PCR using the secondary enrichment.

Table 3.2. Detection of *S. aureus*, *mecA* and PVL genes from animals and retail meat using a conventional culture/PCR method and a real-time PCR assay.

Sample type	No. of samples	Culture/PCR method			Real-time PCR					
					Primary enrichment			Secondary enrichment		
		S. aureus	*mecA*	PVL	*S. aureus*	*mecA*	PVL	*S. aureus*	*mecA*	PVL
Animals		----No. of positives----								
Cow	14	0	0	0	4	0	0	3	0	0
Pig	28	21	0	0	25	0	0	24	1	0
Sheep	35	11	0	0	14	1	0	14	0	0
Total	77	32	0	0	43	1	0	41	1	0
Meat										
Beef	36	9	0	0	10	0	0	12	0	0
Pork	37	25	2	0	26	6	0	27	6	0
Poultry	39	24	0	0	28	0	0	21	0	0
Total	112	58	2	0	64	6	0	60	6	0
Deli meat										
Chicken	9	2	0	0	2	0	0	4	0	0
Ham	20	3	0	0	2	3	0	11	5	0
Turkey	16	0	0	0	0	1	0	4	1	0
Total	45	5	0	0	4	4	0	19	6	0
Total	234	95	2	0	111	11	0	120	13	0

Table 3.3. Raw agreement indices among conventional culture/PCR method and real-time PCR assay, with two-step enrichment procedure for detection of *S. aureus* from animals and retail meat.

Method/sample type	Samples (n)	Culture/ PCR method	Positive agreement (Sensitivity)	Negative agreement (Specificity)	Total agreement	*kappa* statistic
Real-time PCR primary enrichment		No. positive	--------------------No. (%)--------------------			
Animals	77	32	32 (100.0)	34 (75.6)	66 (85.7)	0.72
Meat	112	58	52 (89.7)	42 (77.8)	94 (83.9)	0.68
Deli meat	45	5	4 (80.0)	40 (100.0)	44 (97.8)	0.88
Real-time PCR secondary enrichment						
Animals	77	32	32 (100.0)	36 (80.0)	68 (88.3)	0.77
Meat	112	58	52 (89.7)	46 (85.2)	98 (87.5)	0.75
Deli meat	45	5	5 (100.0)	26 (65.0)	31 (68.9)	0.29

Percentages for positive agreement with culture/PCR method number positive as the denominator. Percentages for negative agreement with culture/PCR method number negative as the denominator. Percentage total agreement is obtained from the sum of the positive and negative agreement frequencies divided by the total sample size within each sample type.

Six samples isolated from animals and six from raw meat were deemed *S. aureus* negative by the conventional culture/PCR method, but positive by real-time PCR using the primary and secondary enrichments. Three *S. aureus* samples isolated from raw meat were positive by the conventional culture/PCR method, but negative by the real-time PCR assay.

The real-time PCR method using the primary enrichment failed to detect the presence of *S. aureus* in four samples: three isolated from raw meat (two from beef, one from poultry) and one from deli meat (ham) that were positive by the culture method and by the real-time PCR assay using the secondary enrichment samples. Using the secondary enrichment samples, the real-time PCR assay failed to detect three samples isolated from raw meat (pork) that were *S. aureus* positive by the culture method and using the primary enrichment in real-time PCR.

The results of real-time PCR using primary and secondary enrichment on the detection of the *mecA* gene compared with a conventional culture/PCR method are shown in Table 3.4. Total agreement for real-time PCR using the primary and secondary enrichments ranged from 91.1% to 98.7% and from 86.7 to 98.7%, respectively. The *k* statistic was zero when the *mecA* gene was not detected by the conventional culture/PCR method and 0.49 ($CI_{95\%}$, 0.39-0.58) for meat. Positive agreement (sensitivity) of 100% was obtained for meat samples for both methods.

Table 3.4. Raw agreement indices among conventional culture/PCR method and real-time PCR assay, with two-step enrichment procedure for detection of the *mecA* gene from animals and retail meat.

Method/sample type	Samples (n)	Culture/ PCR method	Positive agreement (Sensitivity)	Negative agreement (Specificity)	Total agreement	*kappa* statistic
Real-time PCR primary enrichment		No. positive	--------------------No. (%)--------------------			
Animals	77	0	-	76 (98.7)	76 (98.7)	0.00
Meat	112	2	2 (100.0)	106 (96.4)	108 (96.4)	0.49
Deli meat	45	0	-	41 (91.1)	41 (91.1)	0.00
Real-time PCR secondary enrichment						
Animals	77	0	-	76 (98.7)	76 (98.7)	0.00
Meat	112	2	2 (100.0)	106 (96.4)	108 (96.4)	0.49
Deli meat	45	0	-	39 (86.7)	39 (86.7)	0.00

Percentages for positive agreement with culture/PCR method number positive as the denominator. Percentages for negative agreement with culture/PCR method number negative as the denominator. Percentage total agreement is obtained from the sum of the positive and negative agreement frequencies divided by the total sample size within each sample type.

The real-time PCR detected the *mecA* gene in samples that were negative for *S. aureus* by the conventional culture/PCR method (one from a pig, one from a sheep, four from pork meat, four from deli ham, and one from deli turkey). All of these samples were identified as harboring *S. epidermidis*, *S. saprophyticus* or *S. haemolyticus* using biochemical analysis on isolates

recovered. However, three of these samples (one from a pig, and two from pork meat) tested positive for the *nuc* gene when the primary and secondary enrichments were assessed by real-time PCR.

Table 3.5 shows the antimicrobial resistance profiles and the sequence types of the ninety five *S. aureus* strains isolated from animals and retail meat by the conventional culture/PCR method. A total of thirteen antimicrobial resistance profiles were identified among *S. aureus* isolates. Most of the *S. aureus* isolates were resistant to tetracycline and lincomycin, and were ST9. A total of twenty-two *S. aureus* isolates exhibited multidrug resistance. Susceptibility to all antimicrobials tested were found in thirty-five *S. aureus* isolates, which were mostly recovered from chicken meat and identified as ST5.

3.5. Discussion

In this study, a high recovery of *S. aureus* was found in animals and meat samples by the culture/PCR method and the real-time PCR assay (Table 3.2). The inclusion of selective enrichment steps has been found to increase the rate of detection of *S. aureus* (de Boer *et al*., 2009). Waters *et al*. (2011) also found a high prevalence of *S. aureus* in raw meat (47%) using a single step selective enrichment protocol, followed by plating on Baird Parker agar, and confirmation by real-time PCR targeting the *femA* gene.

Table 3.5. Antimicrobial resistance profiles and sequence types of *S. aureus* isolated by conventional culture/PCR method from animals and retail meat.

Antimicrobial resistance profile	No. of antimicrobial subclasses	No. of *S. aureus* isolates with the specific profile	Sequence types (n)†
PEN-TET-ERY-TYL-LINC-STR-CHL	6	2	Pig-ST9 (2)
PEN-TET-LINC-STR-CHL	5	1	Pig-ST9 (1)
TET-ERY-TYL-LINC	3	7	Pork-ST398 (5) Pork-ST5 (1)** Pork-ST9 (1)
PEN-LINC-STR	3	1	Pig-ST9 (1)
TET-ERY-LINC	3	7	Pork-ST9 (4) Pork-ST15 (2) Pork-ST8 (1)
TET-LINC-STR	3	1	Pig-ST9 (1)
ERY-TYL-LINC	2	3	Chicken-ST5 (3)
PEN-ERY	2	3	Pork-ST5 (1) Pork-ST5 (1)** Pork-ST9 (1)
TET-LINC	2	15	Sheep-ST398 (4) Pig-ST9 (11)
ERY-LINC	2	1	Pork-ST9 (1)
TET	1	13	Sheep-ST398 (3) Sheep-ST133 (2) Sheep-ST2111 (1) Pig-ST9 (1) Pork-ST1 (2) Pork-ST5 (2) Pork-ST398 (1) Pork-ST15 (1)
ERY	1	1	Deli chicken-ST39 (1)
LINC	1	5	Pig-ST9 (3) Sheep-ST133 (1) Deli ham-ST15
Susceptible to all tested	0	35	Chicken-ST5 (15) Chicken-ST6 (3) Chicken-ST508 (1) Chicken-NT (1)‡ Pork-ST5 (2) Beef-ST1159 (3) Beef-ST2187 (1) Beef-ST188 (1) Beef-ST15 (1) Beef-ST72 (1) Beef-ST5 (1) Beef-ST1 (1) Deli ham-ST146 (1) Deli ham-ST5 (1) Deli chicken-ST5 (1) Pig-ST9 (1)
Total		95	

Chloramphenicol (CHL); erythromycin (ERY); lincomycin (LINC); penicillin (PEN); streptomycin (STR); tetracycline (TET); tylosin (TYL). †Sequence type (ST). ‡Non-typeable (NT). ***mecA* gene positive.

The *k* statistic for detection of *S. aureus* using the primary enrichment in real-time PCR was 0.68 to 0.88 (Table 3.3), which indicates a good agreement (substantial to almost perfect agreement) with the conventional culture/PCR method. Using the secondary enrichment and real-time PCR, the *k* statistic for detection of *S. aureus* was 0.29 to 0.77, resulting in a fair agreement when deli meat was tested. This is due to the significantly higher recovery of *S. aureus* from the secondary enrichment samples by real-time PCR (Table 3.2), and the lower negative agreement (specificity) obtained with this method (Table 3.3). This observation suggests that small numbers (or levels) of *S. aureus* could be missed when the primary enrichment alone is used in real-time PCR, and that the recovery of potentially injured or non-viable strains appears to be enhanced when a secondary enrichment is applied. The enhanced detection also suggests that the use of a standard culture method or primary enrichment alone could lead to higher false negative results. Therefore, including a secondary selective enrichment step appears to improve the odds of detection of positive *S. aureus* samples.

Multiplex real-time PCR could detect more *S. aureus* positive samples than the conventional culture/PCR method alone. Possible reasons for these discrepant results include: amplification of DNA by the real-time PCR from very low levels of *S. aureus* that were not detectable by the bacteriological methods due to competition or non-viable *S. aureus* in the samples, or false-positive real-time PCR results as a result of cross-reaction rather than false-negative culture results (Anderson and Weese, 2007). However, the possibility that these results are considered as false positives in this study is probably very low, because the gene *nuc*, which was targeted by the real-time PCR assay, has been used for specific detection and identification of *S. aureus* previously (Costa *et al*., 2005; McDonald *et al*., 2005; Thomas *et al*., 2007; Kilic *et al*., 2010). Unfortunately, it was not possible to confirm these results by performing the cultural

method as detection was carried out from DNA extracts only, and the cells had already been inactivated. The inability of real-time PCR to detect three *S. aureus* samples isolated from raw meat that were positive by the culture method is somewhat unsatisfactory, and could be considered as false-negative results.

For detection of *mecA* gene, the *k* statistic for both enrichments in real-time PCR was 0 to 0.49 (Table 3.4). The *k*=0 indicates no agreement beyond that expected by chance, because the real-time PCR assay detected the *mecA* gene probably from bacteria other than *S. aureus* and the culture/PCR method detected the *mecA* gene from DNA extracted from confirmed *S. aureus* strains. However, a few *mecA* positive samples were obtained from animals and meat in this study (Table 3.2). Weese *et al*. (2010) detected a low prevalence of MRSA in samples isolated from retail meat (9.6% in pork, 5.6% in beef, and 1.2% in chicken), using a single-step selective enrichment protocol, followed of plating and biochemical testing.

The detection of the *mecA* gene by the real-time PCR assay in samples that were negative for *S. aureus* by the conventional culture/PCR method may be due to the fact that either coagulase-negative staphylococci and non *S. aureus* species can also carry the *mecA* gene (Ryffel *et al*., 1990; Hagen *et al*., 2005; Higashide *et al*., 2006; Thomas *et al*., 2007). In this study, such samples were identified as *Staphylococcus* spp. positive by biochemical testing. In addition, the *mecA* gene has been found in non-staphylococcal genera, such as: *Proteus vulgaris*, *Morganella morganii*, *Enterococcus faecalis* (Kassem *et al*., 2008) suggesting that its use in a rapid screening technique would need further validation to avoid false-positive MRSA. In this study, the DNA extraction was carried out from selective enrichments, which could contain DNA from coagulase-positive or coagulase-negative staphylococci or non-staphylococcal species that may

carry the *mecA* gene, therefore a positive result for the *nuc* and *mecA* genes does not necessarily indicate the presence of *S. aureus* carrying the *mecA* gene.

None of the samples obtained from animals and retail meat were positive for the PVL gene using both methods the conventional multiplex PCR and the real-time PCR. A similar observation was reported by Weese *et al*. (2010), who also failed to detect PVL positive samples in raw meat in Canada using the real-time PCR technique. The PVL gene encodes the Panton-Valentine leukocidin toxin, which is a virulence factor that have been found in severe cases of CA-MRSA (Baba *et al*., 2002; Dufour *et al*., 2002; Ebert *et al*., 2009).

Decreasing the time of detection of *S. aureus* and MRSA has become an important goal in the microbiological analysis of clinical samples. However, since *S. aureus* ST398, multidrug-resistant *S. aureus* (Table 3.5), and MRSA are present in animals and meat (van Belkum *et al*., 2008; de Boer *et al*., 2009; Guardabassi *et al*., 2009; Persoons *et al*., 2009; O'Brien *et al*., 2012; Buyukcangaz *et al*., 2013), decreasing the time of analysis may allow for prompt action to take place thus reducing the spread of those strains to the food chain. The real-time PCR assay can potentially decrease the total time for detection of *S. aureus* and the presence of the *mecA* gene in animal and meat samples. Using the two-step selective enrichment the total time was <2 days by the real-time PCR method, compared with a total time of 6 to 7 days using the culture method that includes selective enrichments, plating steps, biochemical testing and a conventional multiplex PCR for confirmation. However, the presence of MRSA should be confirmed by a culture method if isolates are required for further analysis. Some real-time PCR assays have been developed for the rapid detection of MRSA from clinical samples (Huletsky *et al*., 2004; Hagen *et al*., 2005; Paule *et al*., 2005; Danial *et al*., 2011). Danial *et al*. (2011) reported that the real-time PCR assay detected 0.7% more MRSA-positive samples than the routine standard Brilliance

Chromogenic MRSA agar culture method in a total time of 8 h. Huletsky *et al*. (2004) detected MRSA directly from clinical specimens containing a mixture of staphylococci in less than 1 h, with a false-positive detection rate of 4.6% for MRSA that was actually MSSA. Paule *et al*. (2005) developed a multiplex real-time PCR that detected the genes *femA* and *mecA* directly from blood culture bottles in 2-3 h, obtaining an indeterminate rate of 0.9% when coagulase-negative staphylococci strains were included.

In conclusion, the application of real-time PCR using selective enrichments appears to improve the detection of *S. aureus* and the *mecA* gene in samples extracted from animals, raw meat, and deli meat. The real-time PCR assay may be recommended as a rapid method to detect *S. aureus* and the *mecA* gene in samples obtained from the meat production chain; however, if further confirmation of MRSA should be required (isolate recovery) then the application of the standard culture method in parallel may be warranted.

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4. CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* FROM HUMANS AND A COMPARISON WITH ISOLATES OF ANIMAL ORIGIN

4.1. Abstract

Different clones of methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus* have been found in humans as well as in animals and retail meat. However, more information about the genetic characteristics and similarities between strains is needed. The aim of this study was to identify and characterize *Staphylococcus aureus* from humans, and to compare their characteristics with isolates of animal origin. A total of 550 nasal swabs were taken from healthy humans, and *S. aureus* was isolated and identified. Positive *S. aureus* isolates were subjected to molecular typing and susceptibility testing. In total, 108 MRSA isolates were recovered from clinical patients in the state of North Dakota; and 133 *S. aureus* isolates from animals and meat previously analyzed. The nasal carriage of *S. aureus* in healthy people was 7.6% and, in general, clones were genetically diverse. None of the *S. aureus* strains obtained from healthy people were *mecA*- or PVL-positive. A total of 105 (97.2%) MRSA isolates from clinical cases harbored the *mecA* gene and 11 (10.2%) isolated from blood stream infections harbored the PVL gene. The most common resistance profile among *S. aureus* from healthy people was penicillin, and from clinical cases were erythromycin-penicillin-ciprofloxacin. The rate of multidrug resistance (MDR) was 70% in humans. Most of *S. aureus* harboring *mecA* and PVL genes were identified as ST5 and ST8, and exhibited MDR. However, *S. aureus* isolates of animal origin used for comparison exhibited a lower rate of MDR. The most common resistance profiles in isolates of animal origin were penicillin-tetracycline and penicillin-tetracycline-erythromycin, in animals and raw meat, respectively. The ST5 was also found in

animals and meat, with ST9 and ST398 being the major clones. The genetic similarity between clones from humans and meat suggests the risk of spread of *S. aureus* in the food chain.

4.2. Introduction

In the last few decades, many bacterial species have developed resistance to antimicrobial agents that have been commonly used to treat them (Swartz, 1997). *Staphylococcus aureus* is one of the pathogens known to rapidly develop resistance to antimicrobial agents as new antibiotics are introduced (Lowy, 2003). Within a couple years after the introduction of penicillin to clinical medicine, the first penicillin-resistant *S. aureus* was discovered. The first methicillin-resistant *S. aureus* (MRSA) strains were identified from clinical specimens in 1961; two years after methicillin was introduced as an antibiotic (Jevons, 1961; de Lencastre *et al*., 2007).

Methicillin-resistant *S. aureus* has been implicated in community-associated (CA-MRSA), healthcare-associated (HA-MRSA), and livestock-associated (LA-MRSA) infections worldwide. In the United States, the nasal carriage of *S. aureus* in humans was 29% (78.9 million people) and that of MRSA approximately 1.5% (4.1 million people) in 2003-2004 (Gorwitz *et al*., 2008). In 2005, there were an estimated 478,000 hospitalizations that corresponded to *S. aureus* infections, approximately 278,000 of those were attributed to MRSA (Klein *et al*., 2007). In addition, an invasive MRSA infection was developed by about 94,000 people, leading to 19,000 deaths. The distribution of these infections were approximately 86% HA-MRSA and 14% CA-MRSA (Klevens *et al*., 2007). However, HA-MRSA clones have been progressively replaced by CA-MRSA strains due to the expanding community reservoir and the increasing influx into the hospital of individuals who harbor CA-MRSA (D'Agata *et al*., 2009; Nimmo *et al*., 2013).

Meat-producing animals have also been identified as carriers of MRSA (van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Persoons *et al*., 2009). Moreover, it has been found that retail

meat can also be contaminated with MRSA (de Boer *et al*., 2009; Pu *et al*., 2009; Buyukcanganz *et al*., 2013). These findings have increased the concern that food may serve as a vehicle for transmission of MRSA to the human population (O'Brien *et al*., 2012).

Resistance to methicillin in *S. aureus* is primarily mediated by the *mecA* gene, which encodes the low-affinity penicillin-binding protein 2a (PBP2a) (Hartman and Tomasz, 1981; Van De Griend *et al*., 2009). Recently, a novel *mecA* homolog gene ($mecA_{LGA251}$ re-named *mecC*) has been detected in *S. aureus* strains from humans and livestock that were phenotypically resistant to methicillin but tested negative for the *mecA* gene. The *mecC* gene exhibits about 70% sequence homology to the *mecA* gene and is located on the staphylococcal cassette chromosome *mec* type XI (type-XI SCC*mec*) (García-Álvarez *et al*., 2011; Ito *et al*., 2012; Laurent *et al*., 2012; Petersen *et al*., 2012). Among the virulence factors, Panton–Valentine leukocidin (PVL) exotoxin encoding gene has been related to most CA-MRSA strains (Baba *et al*., 2002; Dufour *et al*., 2002) that cause severe skin infections and necrotizing pneumonia (Ebert *et al*., 2009).

Different clones of methicillin-susceptible *S. aureus* (MSSA) and MRSA have been found in humans as well as in animals and retail meat. Clones that cause CA-MRSA infections (USA300 and USA400) are different than those causing HA-MRSA infections (USA100 and USA200) (McDougal *et al*., 2003). Some sequence types (ST) associated to HA-MRSA have been determined, such as: ST5, ST8, ST22, ST36, ST45, among others (Deurenberg *et al*., 2007). The sequence types ST30 and ST80 have been associated with CA-MRSA (Stenhem *et al*., 2010) and ST398 has been linked with animals (van Belkum *et al*., 2008; Krziwanek *et al*., 2009). The sequence types ST398 and ST9 have been detected in both animals (pigs) and meat (pork meat), with a genetic similarity between *S. aureus* strains from these different sources (Buyukcangaz *et*

al., 2013). However, the clonal type ST398 has also been detected in human patients (van Belkum *et al*., 2008; Krziwanek *et al*., 2009).

The objective of this study was to identify and characterize *Staphylococcus aureus* isolated from humans, and to compare the molecular characteristics and antimicrobial susceptibility with *S. aureus* isolates from animals and meat.

4.3. Materials and methods

4.3.1. Samples

A total of 550 nasal swab samples were obtained from undergraduate students enrolled in the Department of Veterinary and Microbiological Sciences, North Dakota State University, who were considered as healthy humans. Samples were obtained from plates used in class studies, that were discarded at the end of the study, thus none of the isolates obtained were identifiable by traceback. Samples were collected in the fall semester of 2010 (*n*=231) and in the spring semester of 2011 (*n*=319). In addition, a total of 108 MRSA isolates recovered from clinical cases of MRSA affected by wound and blood stream infections (sepsis, bone, cerobrospinal fluid [CSF], synovial fluid, subdural fluid, tissue, leg ulcer and pleural fluid) were obtained from the North Dakota Department of Health (Bismarck, ND) in the summer of 2010.

A total of 133 *S. aureus* strains isolated from animals (pig, *n*=30; sheep, *n*=26; cattle, *n*=2), raw meat (pork, *n*=35; chicken, *n*=25; beef, *n*=9), and deli meat (ham, *n*=4; chicken, *n*=2) were used to compare the molecular characteristics and antimicrobial susceptibility with *S. aureus* isolates from humans. The *S. aureus* strains of animal origin were isolated and analyzed as previously described by Buyukcangaz *et al*. (2013) (Tables 4.1 and 4.2).

Institutional Review Board (IRB) approval was sought for the human isolates and the study was considered exempt by NDSU IRB. Institutional Animal Care and Use Committe (IACUC) approval was used for the animal work as described previously (Buyukcangaz *et al*., 2013).

Table 4.1. Source and characteristics of *S. aureus* isolates of animal origin used in the study.

Source	*S. aureus* isolates	16S rRNA	*mecA*	PVL
Animals	----------No.----------	-----------------------No.-----------------------		
Sheep	26	26	0	0
Pig	30	30	0	0
Cattle	2	2	0	0
Total	58	58	0	0
Raw meat				
Pork	35	35	5	0
Chicken	25	25	0	0
Beef	9	9	0	0
Total	69	69	5	0
Deli meat				
Ham	4	4	0	0
Turkey	0	0	0	0
Chicken	2	2	0	0
Total	6	6	0	0

Adapted from Buyukcangaz *et al*. (2013).

4.3.2. Culture method

Nasal swabs were taken from healthy humans by using a sterile moistened swab inserted into the nostril, to a depth of approximately 1 cm, and rotated five times. For each subject, both nostrils were sampled using the same swab. Nasal swabs were inoculated onto mannitol salt agar (MSA) plates (Becton, Dickinson and Company [BD], Sparks, MD) and incubated at 37°C for 48 h. All colonies surrounded by yellow zones on MSA after incubation were selected. Colonies with pink or red zones on MSA were excluded.

Table 4.2. Antimicrobial resistance profiles of *S. aureus* isolates of animal origin used in this study.

Antimicrobial resistance profile	Antimicrobial subclasses resistant to	Isolates	Source	Isolates
	----------No.----------	--No.--	-----------------No.-----------------	
ERY-PEN-TET-GEN-CHL-CIP-QUI	7	1	Pork meat	1
ERY-PEN-TET-CHL-CIP-QUI	6	1	Pork meat	1
ERY-PEN-TET-CHL	4	2	Pig	2
ERY-PEN-TET-KAN	4	1	Pork meat	1
ERY-PEN-TET	3	14	Pork meat	11
				3*
PEN-TET-GEN	3	1	Sheep	1
PEN-TET-KAN	3	1	Pork meat	1*
PEN-TET-CHL	3	1	Pig	1
ERY-PEN	2	4	Pork meat	3
				1*
ERY-TET	2	5	Pork meat	5
PEN-TET	2	39	Pig	19
			Sheep	17
			Pork meat	2
			Chicken meat	1

Ciprofloxacin (CIP); chloramphenicol (CHL); erythromycin (ERY); gentamicin (GEN); kanamycin (KAN); quinupristin/dalfopristin (QUI); penicillin (PEN); and tetracycline (TET). ***mecA* positive. Adapted from Buyukcangaz *et al*. (2013), considering the resistance according to CLSI (2012) criteria.

Table 4.2. Antimicrobial resistance profiles of *S. aureus* isolates of animal origin used in this study (continued).

Antimicrobial resistance profile	Antimicrobial subclasses resistant to	Isolates	Source	Isolates
ERY	1	4	Chicken meat	3
			Chicken deli meat	1
PEN	1	21	Pig	7
			Sheep	1
			Pork meat	4
			Beef	4
			Chicken meat	3
			Ham	2
TET	1	10	Sheep	6
			Pork meat	3
			Chicken meat	1

Ciprofloxacin (CIP); chloramphenicol (CHL); erythromycin (ERY); gentamicin (GEN); kanamycin (KAN); quinupristin/dalfopristin (QUI); penicillin (PEN); and tetracycline (TET). **mecA* positive. Adapted from Buyukcangaz *et al*. (2013), considering the resistance according to CLSI (2012) criteria.

All presumptive *S. aureus* colonies were confirmed by biochemical testing using Sensititre Gram Positive ID (GPID) plates (Sensititre®, TREK Diagnostic Systems Ltd., Cleveland, OH), according to the manufacturer's recommendations.

Staphylococcus aureus isolates from healthy humans, and MRSA isolates from clinical cases, were stored at -80°C in brain–heart infusion broth (BD) containing 20% glycerol until use.

4.3.3. Multiplex polymerase chain reaction (mPCR)

Staphylococcus aureus strains from healthy humans and from clinical cases stored at -80°C were recovered to trypticase soy agar (TSA) plates and incubated at 37°C for 18 to 24 h. The extraction of DNA was carried out by suspending one colony in 50 µL of DNase/RNase-free distilled water (Gibco Invitrogen, Grand Island, NY, USA), heating (99°C, 10 min) and

centrifugation (30,000 × *g*, 1 min) to remove cellular debris. The remaining DNA was transferred to a new tube and stored at -20°C until use.

A multiplex PCR assay was used to detect: 16S rRNA (identification of *S. aureus*), *mecA* (associated with methicillin resistance) and PVL-encoding genes (virulence factor) (Table 4.3). Two microliters of the DNA template (described above) was added to a 50 µL final reaction mixture: 1X Go Taq® Reaction Buffer (pH 8.5), 1.25 U of Go Taq® DNA polymerase, 200 µM dNTP (Promega, Madison, WI, USA), and 1 µM of primers (16S rRNA, *mecA*, LukS/F-PV) (Integrated DNA Technologies, Inc., Coralville, IA, USA). The conditions of the PCR reactions were adjusted according to the protocol described by Makgotlho *et al*. (2009) using a thermocycler (Eppendorf, Hamburg, Germany).

The mPCR products (10 µL) were loaded into a 1.5% (wt/vol) agarose gel (Agarose I™) using EzVision One loading dye (Amresco, Solon, OH, USA) and electrophoresis was carried out in 1X TAE buffer at 100 v for 1 h. A molecular weight marker 100-bp ladder (Promega, Madison, WI, USA) and a negative (DNase/RNase-free distilled water) and a positive control (*S. aureus* ATCC 33591; MRSA) were included on each gel. Bands corresponding to each gene were visualized using an Alpha Innotech UV imager (FluorChem™).

All MRSA clinical isolates that were negative for the *mecA* gene by mPCR assay were subjected to the detection of the *mecC* gene (Table 4.3) by PCR according to the protocol described by Stegger *et al*. (2011).

4.3.4. Pulsed-field gel electrophoresis (PFGE)

The PulseNet protocol with minor modifications was used (McDougal *et al*., 2003). Briefly, *S. aureus* strains were recovered from frozen stock to TSA plates and incubated at 37°C for 18 to 24 h. A single colony was inoculated onto a second TSA plate and incubated at 37°C for 18 to 24

h. Colonies were transferred to 5-mL polystyrene round-bottom tubes containing 2 mL of cell suspension buffer (100 mM Tris HCl [pH 8.0], Invitrogen; 100 mM EDTA [pH 8.0], Gibco), adjusting the cell concentrations to an absorbance of 0.9 to 1.1 using a spectrophotometer (Smart SpecTM plus, Bio-Rad Laboratories, USA) at 610 nm. The following steps (plug preparation, lysis, washing, and the *SmaI* enzyme restriction digestion) were performed according to the PulseNet protocol. *Salmonella* Branderup H9812 was used as a DNA marker (Ribot *et al*., 2000).

The electrophoresis was carried out in a Chef Mapper (Bio-Rad Laboratories) PFGE rig, with an initial switch time of 5 s, a final switch time of 40 s, and a total running time of 17 h 45 min. The gels were stained with ethidium bromide (1.5 μg/mL), and then the macrorestriction patterns were visualized using a UVP imager (UVP, Upland, CA).

Macrorestriction patterns of *Staphylococcus aureus* isolates from humans, animals and meat were analyzed using the BioNumerics Fingerprinting software (Ver 6.6 Applied Math, Austin, TX). The similarity index was calculated using the Dice coefficient, a band position tolerance of 1%, and an optimization of 0.5%. The unweighted-pair group method with arithmetic mean algorithm (UPGMA) was used to construct a dendrogram, and clusters were selected using a cutoff at 80% level of genetic similarity (McDougal *et al*., 2003).

4.3.5. Multilocus sequence typing (MLST)

After the construction of the dendrogram (PFGE) containing *S. aureus*, at least one human isolate from each cluster was selected as a representative of the group for MLST analysis. Strains of *S. aureus* from animals and meat were included for comparison and STs were obtained from previous work (Buyukcangaz *et al*., 2013). Sequencing of MLST PCR products of the selected human isolates was carried out at Iowa State University's DNA Sequencing Facility (Ames, IA).

Briefly, *S. aureus* isolates were struck to TSA plates and incubated at 37°C for 18 to 24 h. DNA extraction from cells was carried out using the boiling method as described above.

Internal fragments of the following seven housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*, were amplified (Table 4.3) (Enright *et al*., 2000). All PCR reactions were carried out in 50-µL volumes: 1 µL of DNA template, Taq DNA polymerase (Promega) (1.25 U), 1X PCR buffer (Promega), primers (0.1 µM) (Integrated DNA Technologies, Inc.), and dNTPs (200 µM) (Promega). The PCR conditions were adjusted according to the protocol described by Enright *et al*. (2000) using a thermocycler (Eppendorf). Ten microliters of the PCR products were loaded into 1% agarose gels in 1X TAE with EzVision One loading dye, and electrophoresis was run at 100 V in 1X TAE for 1 h. Images were captured using an Alpha Innotech imager.

The amplicon purification was carried out using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified PCR products were sequenced at Iowa State University's DNA Facility (Ames, IA) using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence data were imported into DNAStar (Lasergene, Madison, WI), trimmed, and aligned to the control sequences (from the MLST site) and interrogated against the MLST database (http://saureus.mlst.net/). Sequence types of selected *S. aureus* isolates were added to the strain information for analysis in BioNumerics software.

Table 4.3. Nucleotide sequence of the primers used in multiplex polymerase chain reaction for detection of 16S rRNA, *mecA*, Panton-Valentine leukocidin, and *mecA*$_{LGA251}$ genes; and multilocus sequence typing analysis for detection of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* genes.

Primer	Oligonucleotide sequence	Amplicon Size (bp)
Staph 756 F	5’-AACTCTGTTATTAGGGAAGAACA-3’	756
Staph 750 R	5’-CCACCTTCCTCCGGTTTGTCACC-3’	
mecA 1 F	5’-GTAGAAATGACTGAACGTCCGATAA-3’	310
mecA-2 R	5’-CCAATTCCACATTGTTTCGGTCTAA-3’	
luk-PV-1 F	5’-ATCATTAGGTAAAATGTCTGGACATGATCCA-3’	433
luk-PV-2 R	5’-GCATCAAGTGTATTGGATAGCAAAAGC-3’	
mecA$_{LGA251}$FP	5'-TCACCAGGTTCAAC[Y]CAAAA-3'	356
mecA$_{LGA251}$RP	5'-CCTGAATC[W]GCTAATAATATTTC-3'	
mecA$_{LGA251}$MultiFP	5'-GAAAAAAAGGCTTAGAACGCCTC-3'	718
mecA$_{LGA251}$RP	5'-CCTGAATC[W]GCTAATAATATTTC-3'	
mecA$_{LGA251}$MultiFP	5'-GAAAAAAAGGCTTAGAACGAATC-3'	138
mecA$_{LGA251}$MultiRP	5'-GATCTTTTCCGTTTTCAGC-3'	
arcC F	5'-TTGATTCACCAGCGCGTATTGTC-3'	456
arcC R	5'-AGGTATCTGCTTCAATCAGCG-3'	
aroE F	5'-ATCGGAAATCCTATTTCACATTC-3'	456
aroE R	5'-GGTGTTGTATTAATAACGATATC-3'	
glpF F	5'-CTAGGAACTGCAATCTTAATCC-3'	465
glpF R	5'-TGGTAAAATCGCATGTCCAATTC-3'	
gmK F	5'-ATCGTTTTATCGGGACCATC-3'	429
gmK R	5'-TCATTAACTACAACGTAATCGTA-3'	
pta F	5'-GTTAAAATCGTATTACCTGAAGG-3'	474
pta R	5'-GACCCTTTTGTTGAAAAGCTTAA-3'	
tpi F	5'-TCGTTCATTCTGAACGTCGTGAA3'	402
tpi R	5'-TTTGCACCTTCTAACAATTGTAC-3'	
yqiL F	5'-CAGCATACAGGACACCTATTGGC-3'	516
yqiL R	5'-CGTTGAGGAATCGATACTGGAAC-3'	

16S rRNA, *mecA*, and Panton-Valentine leukocidin genes (McClure *et al*., 2006).
mecA$_{LGA251}$ gene (Stegger et al., 2011).
arcC, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* genes (Enright *et al*., 2000).

4.3.6. Susceptibility testing

Staphylococcus aureus isolates were subjected to antimicrobial susceptibility testing using the broth microdilution method and the National Antimicrobial Resistance Monitoring System (NARMS) panels (CMV3AGPF, Sensititre®, Trek Diagnostics), according to the manufacturer's and the Clinical Laboratory Standards Institute (CLSI, 2012) guidelines. Antimicrobials in the panel and their resistance breakpoints were as follows: erythromycin (≥8 µg/mL), tetracycline (≥16 µg/mL), ciprofloxacin (≥4 µg/mL), chloramphenicol (≥32 µg/mL), penicillin (≥0.25 µg/mL), vancomycin (≥16 µg/mL), nitrofurantoin (≥128 µg/mL), gentamicin (≥16 µg/mL), quinupristin/dalfopristin (≥4 µg/mL), linezolid (≥8 µg/mL), kanamycin (≥64 µg/mL), and daptomycin (susceptible ≤1 µg/mL). Multidrug resistance (MDR) was considered as resistance to at least three classes of the antimicrobials tested (Aydin *et al*., 2011).

4.4. Results

The results for identification of *S. aureus* (16S rRNA), *mecA* and PVL genes in samples obtained from humans are shown in Table 4.4. The prevalence of nasal carriage of *S. aureus* in healthy people was 7.6%. None of these isolates harbored the *mecA* or PVL genes. Clinical isolates were identified as MRSA strains in the hospital using standard microbiological procedures. As expected, all of these isolates were confirmed as *S. aureus* strains by the detection of 16S rRNA gene using the PCR assay.

Among the 108 MRSA clinical isolates, a total of 105 (97.2%) harbored the *mecA* gene and 11 (10.2%) carried the PVL gene. Of interest, the PCR assay did not detect the PVL gene in MRSA strains isolated from clinical cases affected by wound infections.

Table 4.4. Identification of 16S rRNA, *mecA* and Panton-Valentine Leukocidin (PVL) genes in *S. aureus* from healthy people, and MRSA isolates from clinical cases.

Source	Samples	Positive for *S. aureus*	Positive for MRSA	16S rRNA	*mecA*	PVL
Healthy people	---No.---	-----No.-----	--No. (%)--	----------------No. (%)----------------		
Fall 2010	231	17 (7.4)		17 (7.4)	0 (0.0)	0 (0.0)
Spring 2011	319	25 (7.8)		25 (7.8)	0 (0.0)	0 (0.0)
Total	550	42 (7.6)		42 (7.6)	0 (0.0)	0 (0.0)
Clinical cases						
Blood	99	99 (100)	99 (100)	99 (100)	96 (97.0)	11 (11.1)
Wound	9	9 (100)	9 (100)	9 (100)	9 (100)	0 (0.0)
Total	108	108 (100)	108 (100)	108 (100)	105 (97.2)	11 (10.2)

The genetic similarity between *S. aureus* strains isolated from humans and *S. aureus* strains of animal origin were analyzed using BioNumerics software. Figure 4.1 shows a dendrogram containing the macrorestriction patterns of *S. aureus* strains and the sequence type (ST) of some isolates from each cluster. Thirty-four *S. aureus* ST398 strains of animal origin were not included in the dendrogram as they failed to restrict. A total of fifteen clusters was observed, of which six were homogenous, containing one type of isolate exclusively from healthy humans (cluster 1 and 2), MRSA isolates from clinical cases (cluster 9), or isolates of animal origin (clusters 10, 11 and 15). In general, genetic diversity was observed among isolates from healthy humans, classified in different clusters with the sequence types: ST5, ST15, ST30, ST34, ST39, ST45. Genetic similarity was observed between *S. aureus* strains from humans and meat: cluster 3 (ST39), cluster 4 (ST1), cluster 7 (ST5), and cluster 12 (ST15). In cluster 9, genetic similarity was observed between *mecA*-positive strains and one strain that did not harbor *mecA* nor *mecC* genes isolated from clinical cases, and were identified as ST8. In addition, two clinical isolates

identified as MRSA ST5 that were *mecA*- and *mecC*-negative exhibited a genetic similarity with *mecA*-positive *S. aureus* ST5 strains isolated from humans and from pork meat (cluster 8).

Among the antimicrobials tested using CLSI interpretation criteria (CLSI, 2012), most MRSA isolates from clinical cases were resistant to erythromycin, penicillin and ciprofloxacin, and *S. aureus* isolates from healthy people exhibited resistance primarily to penicillin (Table 4.5). A rate of 70% of MDR strains was detected in humans, primarily among clinical isolates that were all identified as MRSA. In humans, one clinical isolate identified as MRSA was susceptible to all antimicrobial agents. The minimum inhibitory concentrations (MICs) of resistant *S. aureus* strains from humans are shown in Table 4.6. High MICs were observed in most of the penicillin-resistant *S. aureus* isolates from humans (8 - >16 µg/mL). The majority of ciprofloxacin-resistant *S. aureus* isolates from humans exhibited a MIC > 4 µg/mL.

4.5. Discussion

Presumptive *S. aureus* samples on MSA plates from healthy people were confirmed by biochemical testing (Sensititre identification plates) with an agreement of 100% with PCR (detection of the 16S rRNA gene) (Table 4.4). In this study, the nasal carriage of *S. aureus* was 7.6%, which is considerably lower than the prevalence found in other studies (29-32%) (Mainous *et al*., 2006; Gorwitz *et al*., 2008). However, those studies considered a larger sample size, different demographic characteristics, and different sampling years as part of a nationally representative assessment of carriage of *S. aureus*. In this study, *S. aureus* strains isolated from healthy people did not harbor the *mecA* or PVL genes. Other studies have reported a nasal carriage rate of MRSA of approximately 0.8 to 1.5% in the community (Mainous *et al*., 2006; Gorwitz *et al*., 2008), 0.5 to 44% in patients (Tiemersma *et al*., 2004), 20% in healthcare workers (Kumar *et al*., 2011) and 30% in people living and working on farms with MRSA-positive pigs

or dust (Van Den Broek *et al*., 2009). Buyukcangaz *et al*. (2013) failed to detect the *mecA* and PVL genes in *S. aureus* isolates from meat-producing animals (Table 4.1) that were used for the comparison with human isolates in this study. However, a low prevalence of *S. aureus* harboring the *mecA* gene was found in pork meat.

The proportion of MRSA in relation to all *S. aureus* strains causing infections is still unknown, making it difficult to accurately estimate the magnitude of MRSA infections and to design appropriate health action policies (Klevens *et al*., 2007; Moxnes *et al*., 2013). In this study, three clinical isolates identified as MRSA were negative for the *mecA* gene using the protocol described by Makgotlho *et al*. (2009). For that reason, the presence of the novel *mecA* homolog gene ($mecA_{LGA251}$ or *mecC*), was assessed using the protocol described by Stegger *et al*. (2011). However, those strains were also negative for the *mecC* gene (138 and 718 bp fragments), but tested positive por the 356 bp fragment using degenerate primers. Therefore, further investigation should be carried out to determine the genetic variation of this fragment. In addition, it is known that borderline oxacillin-resistant *S. aureus* (BORSA) exhibit an intermediate resistance level to oxacillin, which is non-*mecA* mediated (Nadarajah et al., 2006; Stefani *et al*., 2012). All *mecA*- and *mecC*-negative *S. aureus* strains identified as MRSA were subjected to oxacillin susceptibility testing. One of those isolates exhibited an intermediate resistance level to oxacillin (2-4 µg/mL) (CLSI, 2012), which could be considered as BORSA. Different modifications in the PBP genes causing amino acid substitutions in the transpeptidase domain has been also associated with the borderline resistance (Nadarajah et al., 2006).

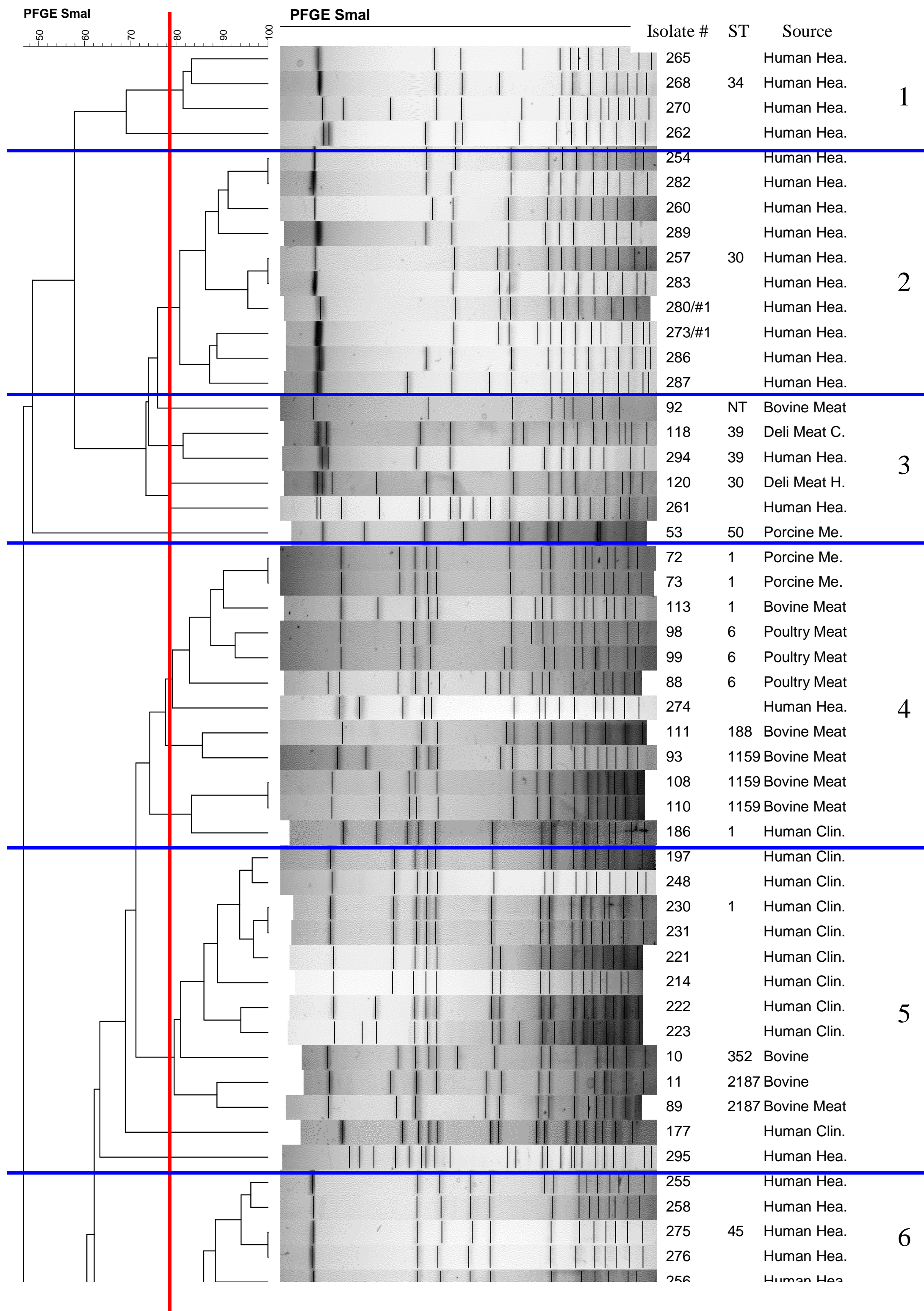


Figure 4.1. Dendrogram showing the genetic similarity between *S. aureus* isolates from humans, and of animal origin. The scale indicates levels of similarity, numbers represent the sample codes, followed on the right by the sequence type (ST) and the type of the sample. **mecA*-positive *S. aureus* in pork meat. ***mecA*- and *mecC*-negative MRSA from clinical cases.

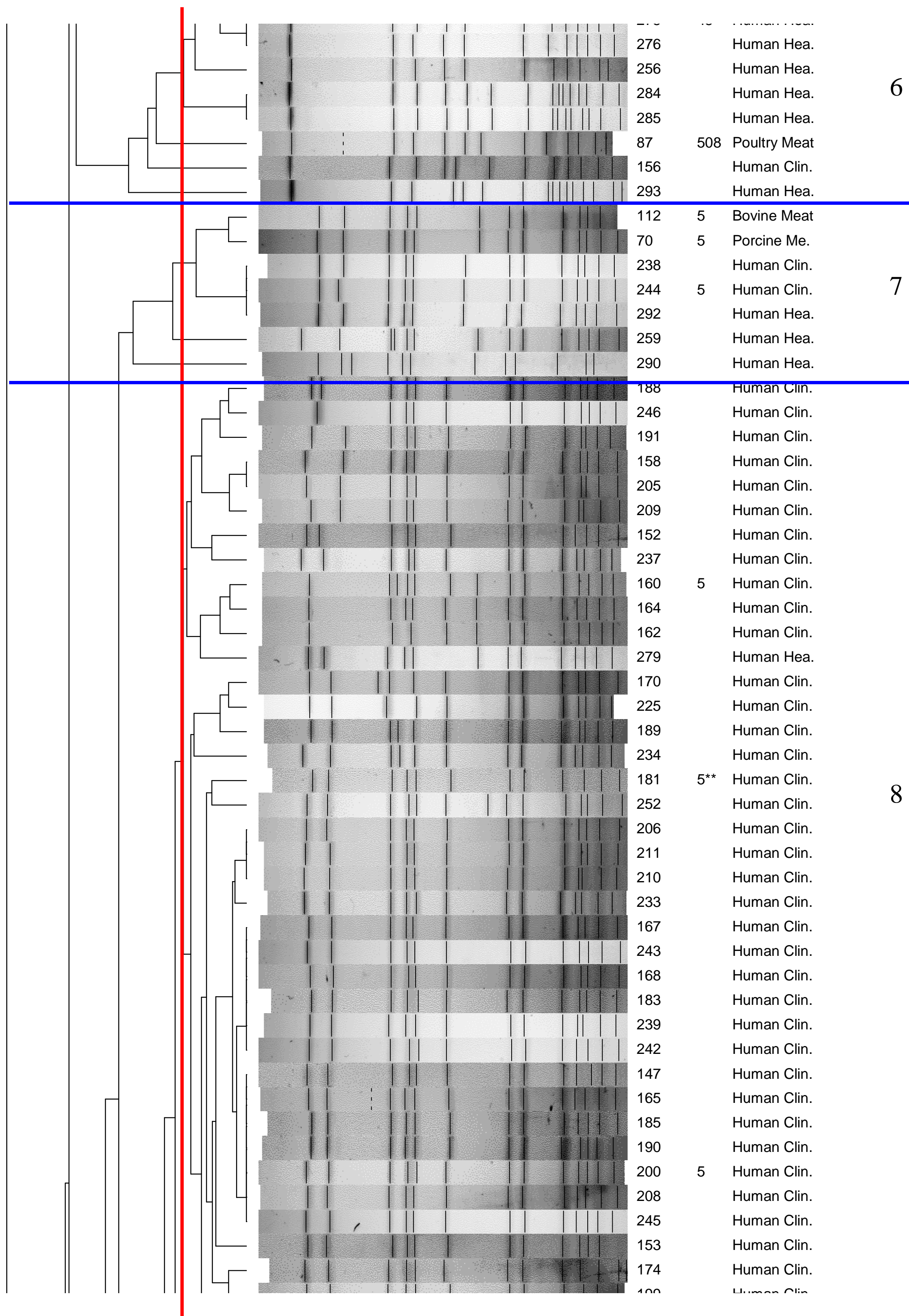
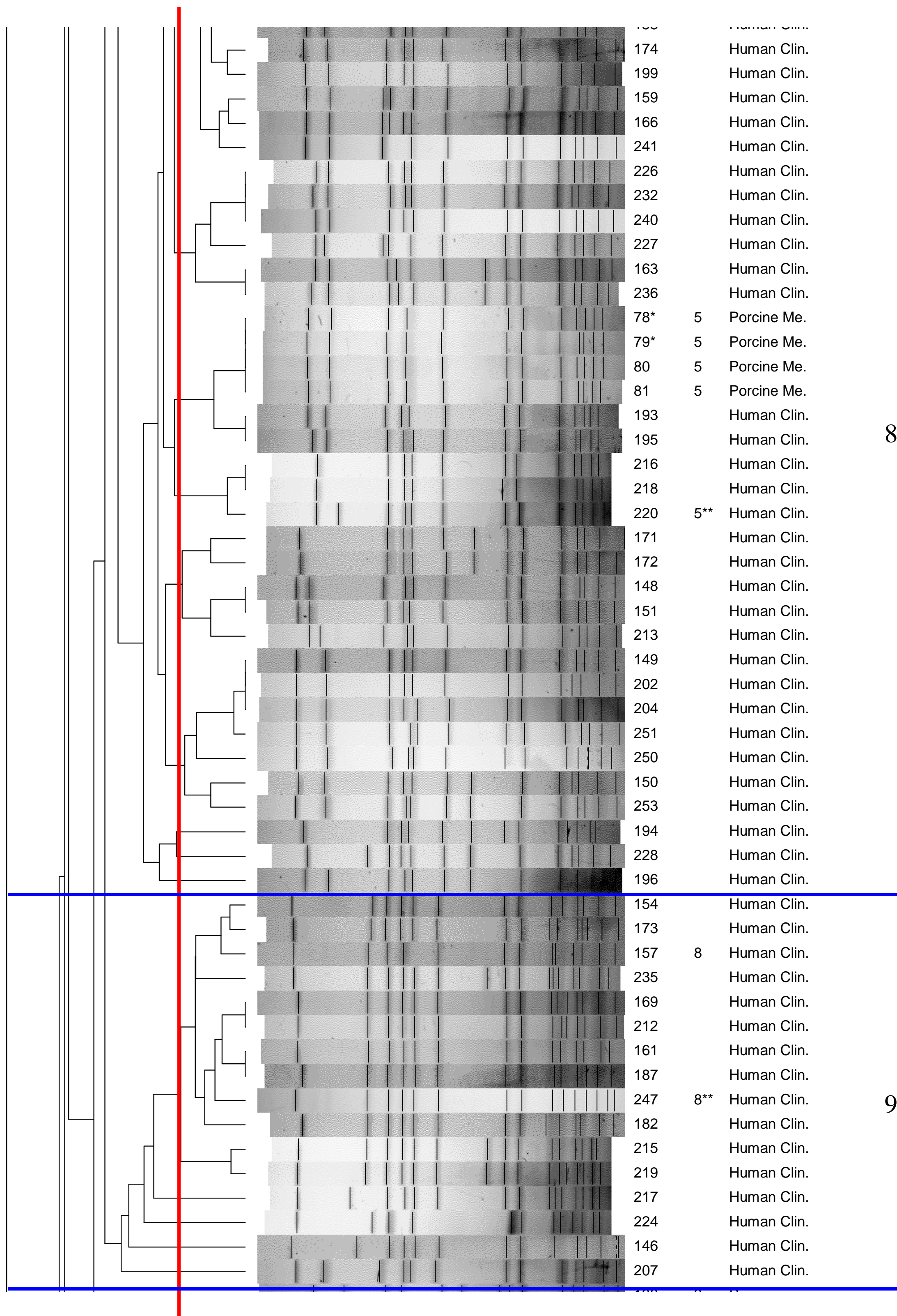


Figure 4.1. Dendrogram showing the genetic similarity between *S. aureus* isolates from humans, and of animal origin (continued). The scale indicates levels of similarity, numbers represent the sample codes, followed on the right by the sequence type (ST) and the type of the sample. **mecA*-positive *S. aureus* in pork meat. ***mecA*- and *mecC*-negative MRSA from clinical cases.



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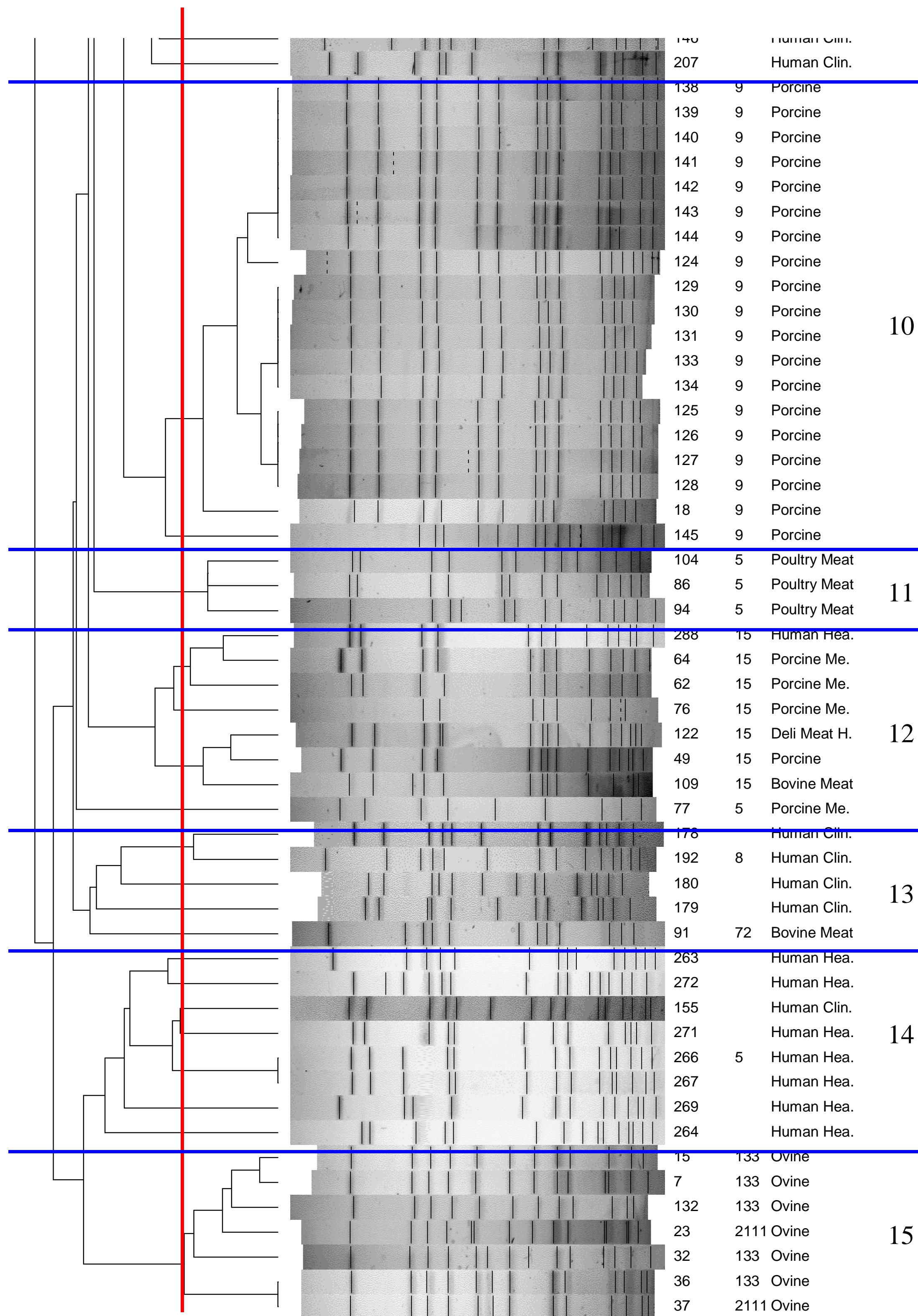

Figure 4.1. Dendrogram showing the genetic similarity between *S. aureus* isolates from humans, and of animal origin (continued). The scale indicates levels of similarity, numbers represent the sample codes, followed on the right by the sequence type (ST) and the type of the sample. **mecA*-positive *S. aureus* in pork meat. ***mecA*- and *mecC*-negative MRSA from clinical cases.

Table 4.5. Antimicrobial resistance profiles of *Staphylococcus aureus* from healthy people, and methicillin-resistance *Staphylococcus aureus* (MRSA) isolates from clinical cases.

Antimicrobial resistance profile	Antimicrobial subclasses resistant to	Samples	Type	Samples
	------------No.-----------	---No.---	--------------No.--------------	
ERY-PEN-TET-CIP-KAN	5	5	Clinical MRSA*	2
			Healthy human	3
ERY-PEN-CIP-KAN-QUI	5	1	Clinical MRSA*	1
ERY-PEN-CIP-KAN-DAP	5	5	Clinical MRSA*	5
ERY-PEN-CIP-KAN	4	29	Clinical MRSA*	25
			Clinical MRSA**	2
			Healthy human	2
ERY-PEN-CIP-DAP	4	1	Clinical MRSA*	1
ERY-PEN-TET	3	2	Healthy human	2
ERY-PEN-KAN	3	3	Clinical MRSA*	2
			Healthy human	1
ERY-CIP-KAN	3	1	Clinical MRSA*	1
PEN-TET-CIP	3	1	Clinical MRSA*	1
PEN-CIP-KAN	3	2	Clinical MRSA*	1
			Healthy human	1
ERY-PEN-CIP	3	55	Clinical MRSA*	51
			Healthy human	4
ERY-PEN	2	9	Clinical MRSA*	3
			Healthy human	6
PEN-CIP	2	8	Clinical MRSA*	3
			Healthy human	5
ERY-CIP	2	1	Clinical MRSA*	1
ERY	1	2	Healthy human	2
PEN	1	22	Clinical MRSA*	6
			Healthy human	16
CIP	1	2	Clinical MRSA*	2

Ciprofloxacin (CIP); Daptomycin (DAP); erythromycin (ERY); kanamycin (KAN); quinupristin/dalfopristin (QUI); penicillin (PEN); and tetracycline (TET). **mecA* positive. ***mecA* and *mecC* negative. Resistance according to CLSI (2012) criteria.

Table 4.6. Minimum inhibitory concentrations (MICs) of resistant *Staphylococcus aureus* isolates from healthy humans and clinical cases.

Antimicrobial Agent (breakpoints)[†]	Resistant *S. aureus* isolates	MIC (μg/mL)													
		0.5 – 1	2	4	>4	8	>8	16	>16	32	>32	256	512	1024	>1024
	----No.----	----No. (%)----													
ERY	114					7	107								
(≥8 μg/mL)						(6.1)	(93.9)								
PEN	143	11	9	26		36		36	25						
(≥0.25 μg/mL)		(7.7)	(6.3)	(18.2)		(25.2)		(25.2)	(17.5)						
TET	8							2		2	5				
(≥16 μg/mL)								(25.0)		(25.0)	(50.0)				
KAN	46											13	10	13	10
(≥64 μg/mL)												(28.3)	(21.7)	(28.3)	(21.7)
CIP	111			1	110										
(≥4 μg/mL)				(0.9)	(99.1)										
QUI	1								1 (100)						
(≥4 μg/mL)															
DAP[‡]	6		3	3											
			(50)	(50)											

Ciprofloxacin (CIP); Daptomycin (DAP); erythromycin (ERY); kanamycin (KAN); quinupristin/dalfopristin (QUI); penicillin (PEN); and tetracycline (TET). [†]Levels of MIC against tested antibiotics (CLSI, 2012). [‡]Criteria for Dap: susceptible ≤1 μg/mL.

The virulence factor PVL, was detected in this study in 11.1% of MRSA isolates from clinical cases identified as blood stream infections. The MRSA isolates from cases identified as wound infections did not harbor the PVL gene. The PVL toxin is a pore-forming protein that appears to be associated with increased disease severity of *mecA*-positive *S. aureus* strains, mainly in blood stream infections (Dufour *et al*., 2002). Although the PVL gene is considered as a stable marker for CA-MRSA, some CA-MRSA strains have been found to be PVL-negative (Nimmo *et al*., 2013).

In this study, some *S. aureus* strains isolated from humans of each cluster in the dendrogram (Figure 4.1) were subjected to MLST to determine the sequence type. In general, different clones were observed in healthy humans, which indicate the presence of genotypically diverse *S. aureus* clones in the community. Although, MRSA strains were not detected in healthy people, they could become carriers with the risk of spreading infections to the community (Kumar *et al*., 2011). Methicillin-resistant *S. aureus* strains isolated from clinical cases in this study presented a lower genetic diversity, and were primarily of ST5 and ST8. Previously, both ST5 and ST8 have been associated with HA-MRSA infections (Deurenberg *et al*., 2007; Stefani *et al*., 2012). The description of the genetic characteristics of MRSA clones that are causing invasive human infections could help to focus efforts to study the most common clones. The molecular characteristics of *S. aureus* strains isolated from humans were compared with isolates of animal origin. A genetic similarity was observed between *mecA*- and *mecC*-negative MRSA isolates from clinical cases and *mecA*-positive *S. aureus* strains isolated from clinical cases and pork meat, which could be due to modifications in the PBP genes in *mecA*- and *mecC*-negative MRSA strains (Nadarajah *et al*., 2006) that result in slight changes in the macrorestriction patterns. Contamination of meat with *S. aureus* strains from animals and humans could occur during

slaughtering or processing. In this study, a genetic similarity between strains from humans and meat may suggest the contamination of raw meat during handling. In addition, the genetic similarity of *S. aureus* strains isolated from meat-producing animals and retail meat has been found previously, also suggesting the contamination of meat during slaughtering (Buyukcangaz *et al*., 2013). In this study, other *S. aureus* strains that have been previously related to LA-MRSA and pig farmers, such as ST398 and ST9 (van Belkum *et al*., 2008; Buyukcangaz *et al*., 2013; Krziwanek *et al*., 2009) were not detected in *S. aureus* isolates from humans. However, Sung *et al*. (2008) found that animal lineages were closely related to human lineages, which could be due to the adaptive behavior of *S. aureus* (McCarthy *et al*., 2012).

In this study, resistance to penicillin predominated in the *S. aureus* strains isolated from healthy people, and the resistance profiles Ery-Pen-Cip and Ery-Pen-Cip-Kan were most common in MRSA isolates from clinical cases (Table 4.5). Comparing the antimicrobial resistance patterns of human isolates with *S. aureus* isolates of animal origin, some differences were observed (Table 4.2). According to the CLSI (2012) interpretation criteria, most *S. aureus* isolates from animals exhibited resistance to penicillin and tetracycline, and from retail meat to the former antibiotics and erythromycin. Tetracycline-resistant *S. aureus* strains were isolated from animals and retail meat, however, ciprofloxacin-resistant *S. aureus* strains were found in clinical isolates. A higher rate of MDR *S. aureus* strains were obtained from humans than animals and meat, which could be due to the high number of MRSA strains from clinical cases affected by acute infections that were included in this study. Most MRSA strains isolated from clinical cases have been found to be MDR (Arora *et al*., 2014). In addition, clinical isolates (identified as MRSA) showed higher MICs to penicillin (Table 4.6) than *S. aureus* strains obtained from animals and meat (Buyukcangaz *et al*., 2013) suggesting the potential influence of

treatment or exposure on the selection of resistant strains. In this study, all *S. aureus* strains were susceptible to linezolid, which has been considered as a good alternative for the treatment of MDR *S. aureus* (Kishore *et al*., 2014). The CLSI (2012) criteria establishes the susceptibility to daptomycin at MICs ≤1 µg/mL, therefore in clinical isolates MICs of 2 and 4 µg/mL were considered non-susceptible isolates. The interpretation of results for gentamicin, kanamycin, and penicillin could be ambiguous due to their breakpoints. For example, all *S. aureus* strains isolated from humans exhibited MICs ≤128 µg/mL for gentamicin, which has a breakpoint ≥16 µg/mL. For kanamycin and penicillin some *S. aureus* strains showed MICs ≤128 µg/mL and ≤25 µg/mL, respectively, however the CLSI criteria recommends a breakpoint ≥64 µg/mL and ≥0.25 µg/mL as resistance, respectively and our data is limited by the dilution ranges on the current NARMS panel. Therefore, it should be recomended to include a wider range of dilution of antimicrobials on the panel in order to improve the interpretation of susceptibility testing results for those antibiotics.

4.6. Conclusion

The nasal carriage of *S. aureus* in healthy humans appears to be low, with clones genotypically diverse, and were *mecA*- and PVL-negative. *Staphylococcus aureus* strains harboring the *mecA* and PVL genes were present in clinical isolates from patients affected by invasive infections, and most of these isolates were of ST5 and ST8, and exhibited MDR profiles. A genetic similarity between *S. aureus* strains isolated from humans and raw meat suggests that the contamination of meat during handling or processing could be a risk for transmission to humans.

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5. GENERAL DISCUSSION AND OVERALL CONCLUSION

5.1. General discussion

In this study, a high prevalence of *S. aureus* was detected in animals and raw meat (34.7% and 47.6%, respectively), with a higher prevalence in sheep and pigs, and in raw chicken and pork meat. However, the prevalence of *S. aureus* in deli meat was lower (13%). In healthy people, a low *S. aureus* nasal carriage was detected (7.6%) compared with the results from other studies (Mainous *et al*., 2006; Gorwitz *et al*., 2008). In addition, the *mecA* gene was detected only in pork meat, with a low prevalence (3.4%), this data is similar to results reported in other studies (de Boer *et al*., 2009; Pu *et al*., 2009). Also, MDR and ST398 *S. aureus* strains were detected in animals (pig and sheep) and in pork meat. In this study, the PVL-encoding gene was not detected in *S. aureus* isolated from animals, meat or healthy people. The presence of MRSA, MDR and ST398 *S. aureus* strains in meat-producing animals, in retail raw meat and deli meat, suggests the potential exposure of humans to emerging *S. aureus* strains through the meat production chain, with a higher risk in ready-to-eat food. Three MRSA strains isolated from clinical cases did not harbor the *mecA* or *mecC* genes. However, those strains resulted positive for the 356 bp fragment, which could suggest a genetic variation of the gene. The presence of borderline resistance, called as borderline oxacillin-resistant *S. aureus* (BORSA), could also explain the phenotypical resistance to methicillin in MRSA strains that are *mecA*- and *mecC*-negative. Some MRSA strains obtained from clinical cases affected by invasive infections were PVL-positive (10%), all obtained from blood stream infections. The presence of this exotoxin has been associated with increased virulence of *S. aureus* strains, primarily CA-MRSA strains due to the pore formation in the membranes of cells (Dufour *et al*., 2002).

The isolation and identification of *S. aureus* in animals and meat determined by the culture method and biochemical testing agreed with the detection of the 16S rRNA gene by the PCR assay. Therefore, using a selective enrichment step followed by a selective plating step for isolation of *S. aureus*, with biochemical testing for confirmation of presumptive strains, results in an accurate method for detection. In this study, the identification of MRSA was carried out by the detection of the *mecA* gene using the PCR tecnique, and by determining the resistance to β-lactam antibiotics using the antimicrobial susceptibility testing. Other methods have been used for confirmation of MRSA, such as: susceptibility to oxacillin or cefoxitin (Danial *et al*., 2011; Kumar *et al*., 2011; Kim *et al*., 2013; Nimmo *et al*., 2013), and detection of the protein PBP2a by agglutination assay (Anderson and Weese, 2007; Weese *et al*., 2010; Danial *et al*., 2011). The method used for isolation of *S. aureus* and MRSA may affect overall prevalence results. Some authors have used a unique plating step (Weese *et al*., 2006; Aydin *et al*., 2011), others have included selective enrichment steps before the plating step (Wertheim *et al*., 2001; de Boers *et al*., 2009; Pu *et al*., 2009; Tenhagen *et al*., 2009; Broens *et al*., 2011; Pu *et al*., 2011; Zhang *et al*., 2011). de Boer *et al*. (2009) included primary and secondary enrichments similar to those used in this study and obtained a higher detection rate of MRSA in meat samples. Both MSSA and MRSA strains can be isolated using media with sodium chloride. However, some MRSA strains do not grow at concentrations of NaCl higher than 2.5% (Jones *et al*., 1997). In addition, some media supplemented with antibiotics may cause breakthrough growth of MSSA strains (Böcher *et al*., 2008) and also may fail to recover MRSA. Therefore, an antibiotic-free medium should be included even if the objective is recovering MRSA only (Pu *et al*., 2009).

The rapid detection of *S. aureus* and MRSA in animals and meat may allow regulatory authorities to take prompt action in order to decrease the risk of exposure of humans. In this

study, a multiplex real-time PCR assay was developed in order to decrease the time of detection. The real-time mPCR assay targeted the genes: *nuc* (identification of *S. aureus*), *mecA* (associated with MRSA) and PVL (virulence factor), in samples from animals and meat. A high total agreement was found between the conventional culture method and the real-time PCR assay, with a higher detection rate when the secondary enrichment was analyzed. This may due to the recovery of injured cells when the secondary enrichment is used, and the failure of detection of low levels of *S. aureus* when the primary enrichment alone is used in real-time PCR. However, the conventional culture method is considered as the standard method for identification of *S. aureus* (Huletsky *et al*., 2004; Paule *et al*., 2005; Danial *et al*., 2011) and conventional PCR (detection of *mecA* gene) for identification of MRSA (Maes *et al*., 2002; Makgotlho *et al*., 2009). The enrichment broths from animal and meat samples were used to obtain DNA for the real-time PCR assay, in which the concentration and the source of the genetic material were variable. The *mecA* gene was detected in samples that were *S. aureus* negative but *Staphylococcus* spp. positive by the culture method and biochemical testing. These results could be considered as false-positive, because the *mecA* gene has been detected in both *S. aureus* and coagulase-negative staphylococci (Ryffel *et al*., 1990; Hagen *et al*., 2005; Higashide *et al*., 2006; Thomas *et al*., 2007; Black *et al*., 2011). Unfortunately, in this study, the results could not be validated using the culture method, since DNA extracts were used and the cells were previously inactivated. The sensitiveness of detection of the real-time PCR assay may be determined, quantifying the minimum DNA concentration for amplification. The inclusion of an internal amplification control in addition of the external controls, allows the detection of false-negative results caused by inhibitors, thermocycler malfunction, low activity of the polymerase or incorrect PCR solution (Hoorfar *et al*., 2004).

The genetic similarity between *S. aureus* from pigs and pork meat (ST9) and from humans and pork meat suggests the likely meat contamination during slaughtering and processing. Five *S. aureus* isolates from pork meat were positive for the *mecA* gene, of which two were ST5 and three ST398. In addition, a high prevalence of MSSA ST398 was detected in samples of animal origin. Therefore, the presence of the emerging ST398 clones in the meat production chain suggests a potential risk of transmission to humans. The genetic similarity between *mecA*- and *mecC*-negative MRSA isolates from clinical cases and *mecA*-positive *S. aureus* strains isolated from clinical cases and pork meat, could be due to modifications in the PBP genes in *mecA*- and *mecC*-negative MRSA strains (Nadarajah *et al*., 2006) that result in slight changes in the macrorestriction patterns. The genotyping of *S. aureus* strains was carried out by PFGE according the PulseNet protocol, which uses the restriction enzyme *Sma*I. However, the DNA of ST398 strains cannot be digested with *Sma*I, due to the presence of a methylation enzyme, which methylates the *Sma*I-recognition site (Bens *et al*., 2006). Therefore, it is not possible to obtain a macrorestriction pattern for ST398 strains by PFGE using *Sma*I; for this reason, this study used a second restriction enzyme, *Xma*I, an isoschizomer of *Sma*I. However, macrorestriction patterns of ST398 strains with weak bands were obtained using this enzyme making comparative analysis difficult. Other restriction enzymes with different recognition sites could be tested in order to establish a PFGE protocol that allows an accurate analysis of molecular typing of *S. aureus* ST398 and strains with undefined macrorestriction patterns. The molecular typing technique with the highest discrimination power is PFGE (McDougal *et al*., 2003). However, at least two molecular typing methods should be used in order to obtain a higher accuracy in sub-typing strains (Tenover *et al*., 1994). In this study, PFGE and MLST were used for sub-typing *S. aureus* strains. In general, both methods classify the strains in similar clusters (Catry *et al*., 2010).

Another typing method used in *S. aureus* is *spa*-typing, however, similar *spa* types could be obtained in unrelated clonal lines, which is a disadvantage of this technique (Van den Broek IV *et al*., 2009; Golding *et al*., 2008). For that reason, a disagreement between *spa*-typing and PFGE and MLST results could be obtained (Golding *et al*., 2008).

All *mecA*-positive *S. aureus* strains detected in this study were resistant to penicillin, with a higher MIC than other strains. In addition, a high prevalence of MDR *S. aureus* strains was determined. The most common resistance profile in *S. aureus* isolated from animals was penicillin-tetracycline and from meat penicillin-tetracycline-erythromycin. The multidrug resistance was primarily detected in ST398 and ST9 strains, which have been associated with animals, particularly to pigs (Lewis *et al*., 2008; van Belkum *et al*., 2008; Guardabassi *et al*., 2009; Krziwanek *et al*., 2009). In healthy humans, the most common resistance profile observed among *S. aureus* was penicillin, and in clinical cases erythromycin-penicillin-ciprofloxacin. Most MRSA strains isolated from clinical cases have been found to be MDR (Arora *et al*., 2014). Antibiotics are effective drugs against infectious agents, however, the misuse of these drugs could lead to the acquisition of antimicrobial resistance in bacteria, which could take place in health care settings, in the community and in livestock. The extensive use of antimicrobial agents exerts a selective pressure on AR strains, eliminating the susceptible strains (Swartz, 1997; Marinelli and Tomasz, 2010). Glycopeptides, such as vancomycin, are frequently used to treat MRSA infections. However, in recent years the incidence of *S. aureus* with full and intermediate resistance to vancomycin has increased (Tiwari and Sen, 2006). In this study, all *S. aureus* strains of animal origin were susceptible to vancomycin, as well as to daptomycin, linezolid, and nitrofurantoin, which agrees with the results obtained by Pu *et al*. (2011) in meat samples. However, there are some limitations of the use of nitrofurans in food-producing animals, due to

concerns regarding their carcinogenicity (FDA, 2014). In animal husbandry, antibiotics are used for treatment, prevention and growth promotion (DuPont and Steele, 1987; Franco *et al*., 1990). This may create AR pathogens in livestock, with the potential risk of spreading the resistance-related genes in bacteria present in humans. In this study, the detection of MDR *S. aureus* strains in animals and meat suggests the risk of exposure of the human population through the meat production chain. Therefore, some antimicrobial agents could exhibit a limited efficacy for treatment of human diseases (Smith *et al*., 2002).

An action against the spread of AR strains is the reduction of the use of antibiotics in the clinical setting. However, AR genes related to a drug may be transferred to plasmids harboring other AR genes related to antimicrobials used as alternatives to the original drug (Swartz, 1997). The efforts to reduce the use of antibiotics, to ensure the control of infections and surveillance, to select doses and drug combinations, should be increased in order to avoid the emergence of AR strains (Marinelli and Tomasz, 2010). Nowadays, new antimicrobial agents are being developed in order to increase their effectiveness against AR pathogens. Research related to antimicrobial resistance may consider the assessment of susceptibility of AR pathogens to the new antimicrobials and the combinations of them. In addition, information about different resistance mechanisms that pathogens exhibit should be expanded. Thus, the information to control the spread of AR pathogens and to decrease the exposure of humans could be available in the near future. In the United States, the National Antimicrobial Resistance Monitoring System (NARMS), created in 1996 with the collaboration of FDA, CDC, USDA, and state and local public health departments, is in charge of the monitoring of antimicrobial susceptibility of enteric bacteria isolated from humans, meat, and food-producing animals. The pathogens included in NARMS are: *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., *Shigella* spp., and

Enterococcus spp. (FDA, 2012). However, the evidence of the presence of emerging *S. aureus* strains (MRSA, ST398, and MDR) in the meat production chain may be considered in order to extend the monitoring to other AR pathogens beyond the enteric bacteria. Thus, including *S. aureus* in this national monitoring system may allow to know the annual prevalence of AR strains, their antimicrobial susceptibility, susceptibility to new drugs, and to establish control and mitigation strategies with the purpose of decreasing the exposure of humans.

5.2. Overall conclusion

The overall conclusions of this study are presented below:

There is a high prevalence of *S. aureus* in meat-producing animals and raw meat, and a low prevalence in healthy people. *Staphylococcus aureus* strains isolated from animals, meat and healthy people did not harbor the PVL-encoding gene. The *mecA* and PVL-encoding genes were present in *S. aureus* strains isolated from clinical cases affected by invasive infections, with a high prevalence of MDR strains. Although, the prevalence of MRSA in raw meat is low, the high prevalence of MDR and ST398 *S. aureus* strains in the meat production chain suggests a potential risk for transmission to humans. In addition, the genetic similarity between *S. aureus* strains from animals and meat, and from humans and meat, suggests the contamination of meat during slaughtering and processing.

It is recommended to include the detection and the antimicrobial susceptibility testing of MRSA and MDR *S. aureus* strains in the federal surveillance systems, such as NARMS, including the monitoring of the meat production chain, hospital patients and healthy people in the community.

The use of selective enrichments prior to the culture method allows the isolation of *S. aureus* and MRSA, with an agreement of 100% with the conventional PCR technique. The use of

a secondary enrichment with real-time PCR increased the sensitivity of *S. aureus* detection in animal and meat samples. The real-time PCR technique allows rapid detection of *S. aureus* and the *mecA* gene in animal and meat samples, however, the confirmation of MRSA strains should be carried out by the standard culture method if further analysis is required.

It is necessary to determine the molecular characteristics (*mecA*, *mecC*, PVL, other resistance- and virulence factors-encoding genes), the molecular typing (PFGE and MLST) and the antimicrobial susceptibility of AR *S. aureus* strains in order to establish effective control actions to avoid the spread of those strains.

5.3. References

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