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Detección y tipificación molecular de *Staphylococcus aureus* y *Staphylococcus aureus* resistente a meticilina (SARM) en Dakota del Norte, Estados Unidos

Detection and molecular typing of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in North Dakota, United States

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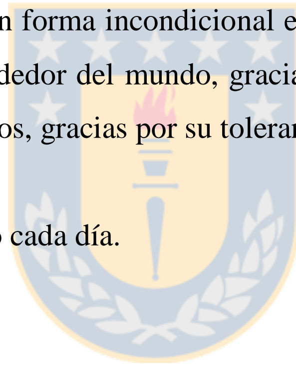
Y a la fuerza divina que recibo cada día.



PRÓLOGO

Esta investigación fue desarrollada en North Dakota State University, Fargo, Dakota del Norte, Estados Unidos durante los años 2010 y 2011.

El escrito de esta tesis consta de una parte general en idioma español (Introducción General, Discusión General y Conclusiones Generales) y tres capítulos en inglés (Desarrollo 1., 2. y 3.) que corresponden a tres artículos científicos. El formato de cada capítulo corresponde al formato exigido por cada revista. La numeración de tablas y figuras se inicia en cada capítulo para mantener el orden de citación dentro de cada artículo.

Las abreviaciones en español e inglés que aparecen en el texto se encuentran en el Glosario de Abreviaciones.

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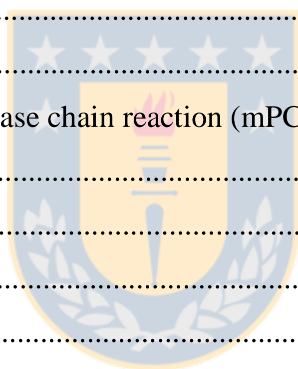

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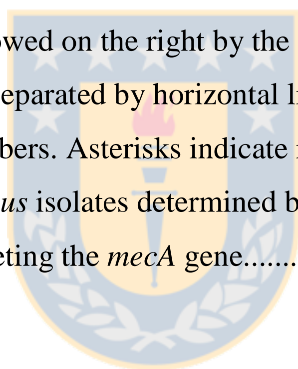

GLOSARIO DE ABREVIACIONES

AR: Antimicrobial-resistant

BP: Baird Parker

CA-MRSA: Community-associated MRSA

CDC: Centers for Disease Control and Prevention

CHL: Chloramphenicol

CIP: Ciprofloxacin

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 resistance (resistant)

MHB: Mueller-Hinton broth

MIC: Minimum inhibitory concentration

MLST: Multilocus sequence typing

mPCR: Multiplex PCR

MR: Multirresistente (resistencia a múltiples agentes antimicrobianos)

MRSA: Methicilin-resistant *S. aureus*

MSSA: Methicillin-susceptible *S. aureus*

NARMS: National Antimicrobial Resistance Monitoring System

QUI/DAL: Quinupristin/dalfopristin

PBP2a: Proteína de unión a la penicilina alterada

PCR: Polymerase chain reaction

PCRm: PCR múltiple

PEN: Penicillin

PFGE: Pulsed-field gel electrophoresis

PHMB^{+}: Phenol red mannitol broth + ceftizoxime + aztreonam

PVL: Leucocidina de Panton-Valentine

SARM: *Staphylococcus aureus* resistente a meticilina

SB: Sheep blood

SCC*mec*: Cassette cromosómico estafilocócico *mec*

ST: Serotipo

STR: Streptomycin

TET: tetracycline

TSA: Trypticase soy agar

USDA: U.S. Department of Agriculture

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RESUMEN GENERAL

La emergencia y la propagación de patógenos resistentes a agentes antimicrobianos ha aumentado la preocupación en materia de salud pública a nivel mundial. El uso de antibióticos en producción animal, en profilaxis, terapia y promoción del crecimiento, se asocia a la emergencia de patógenos resistentes en el ganado. La exposición del ganado a dosis subterapéuticas de agentes antimicrobianos genera un reservorio de patógenos resistentes en los animales con el riesgo de transmisión a las personas a través de la cadena productiva de la carne. Uno de los patógenos que desarrolla rápidamente resistencia a múltiples agentes antimicrobianos (MR) es *Staphylococcus aureus*. En especial, las cepas de *S. aureus* resistente a meticilina (SARM) constituyen una causa importante de infecciones asociadas a hospitales (HA-MRSA), a la comunidad (CA-MRSA) y al ganado (LA-MRSA). La capacidad de colonizar las fosas nasales y piel de humanos y animales que posee *S. aureus* y la detección de cepas de SARM y *S. aureus* MR en animales de abasto y en carne, aumentan la probabiliadad de contaminación de los alimentos en las diferentes etapas de la cadena productiva. La resistencia a la meticilina se atribuye a la proteína de unión a la penicilina 2a (PBP2a) codificada en el gen *mecA*, que forma parte del casssette cromosómico estafilocócico *mec* (SCC*mec*). Esta proteína le confiere a las cepas de SARM una baja afinidad por antibióticos β-lactámicos. A su vez, las cepas CA-MRSA produccen generalmente la exotoxina Leucocidina de Panton-Valentine (PVL), la cual constituye un factor de virulencia asociado a infecciones de la piel y necrosis de tejidos. Por lo tanto, debido al impacto que pueden tener estas cepas de *S. aureus* en la salud humana, resulta relevante evaluar si los productos alimenticios pueden ser un foco de contaminación que facilite la propagación de estas infecciones.

El objetivo de este estudio fue establecer la prevalencia y los tipos moleculares de *S. aureus* y SARM en animales de abasto, carne cruda, productos cárnicos y humanos, determinando la relación filogenética entre las cepas.

Se aisló *S. aureus* desde 167 muestras nasales de animales, 145 muestras de carne cruda y 46 muestras de productos cárnicos, utilizando una etapa de enriquecimiento selectivo en dos pasos, seguido de cultivo en placa. Los aislados que resultaron ser positivos se sometieron al análisis por PCR múltiple (PCRm) para identificar los genes: ARNr 16S (identificación de *S. aureus*), *mecA* (detección de SARM) y PVL (factor de virulencia asociado a CA-MRSA). Para la tipificación molecular de las cepas de *S. aureus* se utilizó electroforesis en gel de campo pulsado (PFGE) y tipificación de secuencias multilocus (MLST). El análisis de susceptibilidad antimicrobiana se llevó a cabo a través del método de microdilución en caldo. Además, se aisló e identificó *S. aureus* desde muestras de fosas nasales de 550 personas sanas a través de métodos de cultivo y análisis bioquímico. Un total de 108 aislados clínicos de cepas SARM obtenidos de pacientes hospitalizados se analizaron a través de PCRm para confirmar la presencia de *S. aureus* y detectar las cepas con los genes *mecA* y PVL. Se desarrolló un método de PCR múltiple en tiempo real con el fin de disminuir el tiempo de análisis de muestras de animales y carne y para aumentar la sensibilidad de detección de *S. aureus* y de los genes *mecA* y PVL. Este procedimiento se realizó con posterioridad al enriquecimiento primario y al secundario que preceden al cultivo en placa. Los resultados se compararon con el método de cultivo que incorpora el enriquecimiento selectivo en dos pasos para aislar *S. aureus*. Se analizó un total de 234 muestras a través de estos métodos (77 muestras nasales de animales, 112 muestras de carne cruda y 45 muestras de productos cárnicos).

La prevalencia total de *S. aureus* determinada luego del análisis de las muestras por el método de cultivo con doble enriquecimiento fue de 34,7% en animales, siendo más alta en cerdos (50,0%) y corderos (40,6%) (P<0,05). En carne cruda la prevalencia fue de 47,6%, siendo más alta en carne de pollo (67,6%) y de cerdo (49,3%) (P<0,05). En productos cárnicos la prevalencia fue de 13,0% (Figura 1). Cinco muestras de carne de cerdo (7,0%) fueron positivas para el gen *mecA*, de las cuales tres correspondieron al serotipo ST398 y dos al serotipo ST5. Todas estas cepas exhibieron resistencia a la penicilina y cuatro fueron MR. En ninguna de las muestras se detectaron a través de PCRm los genes PVL. Los serotipos más

comunes en corderos fueron ST398 y ST133, en cerdos y carne de cerdo ST398 y ST9 y en carne de pollo ST5. Se determinó una similitud genética entre las cepas de *S. aureus* ST9 aisladas de cerdos y carne de cerdo. La mayoría de las cepas de *S. aureus* susceptibles a agentes antimicrobianos fueron ST5 aisladas de carne de pollo. Los aislados MR fueron encontrados en cerdos, carne de cerdo y corderos.

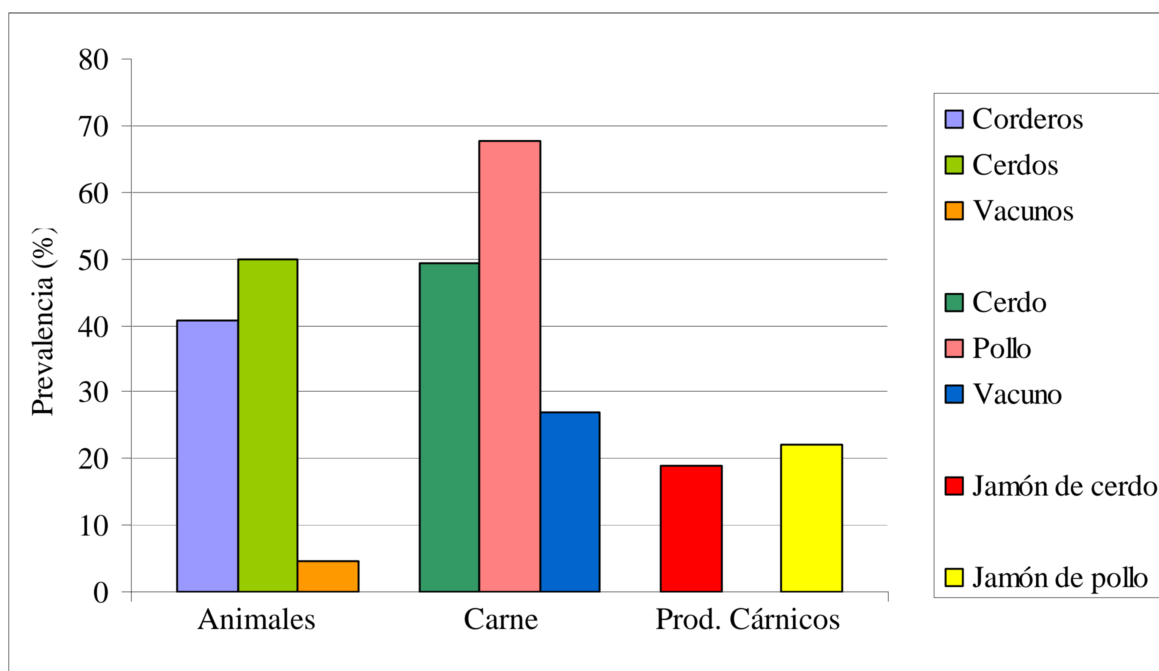

Figura 1. Prevalencia de *Staphylococcus aureus* en animales, carne cruda y productos cárnicos.

La proporción de personas portadoras sanas de *S. aureus* en las fosas nasales fue de 7,6%. Ninguna de estas cepas fue positiva para los genes *mecA* y PVL. Un total de 105 cepas de SARM (97,2%) obtenidas de pacientes hospitalizados presentaron el gen *mecA* y 11 (10,2%) los genes PVL.

La aplicación del método de PCR en tiempo real en muestras con enriquecimiento primario y secundario permitió detectar *S. aureus* en 111/234 y 120/234 muestras, respectivamente. Estos niveles de detección fueron mayores que los obtenidos con el método de cultivo, el cual permitió detectar 95/234. Para la detección de *S. aureus* a través de PCR en tiempo real, la concordancia total con el método de cultivo fue de 83,9-97,8% (*kappa*=0,68-0,88 [considerable a casi perfecta]) y 68,9-88,3% (*kappa*=0,29-0,77 [pobre a considerable]) para el enriquecimiento primario y secundario, respectivamente. Para la detección del gen

mecA, la concordancia total fue de 91,1-98,7% y 86,7-98,7% (*kappa*=0-0,49 [no concordancia más allá del azar a moderada]) para el enriquecimiento primario y secundario, respectivamente. El método de PCR en tiempo real detectó el gene *mecA* en algunas muestras que fueron negativas para *S. aureus*, pero positivas para *Staphylococcus* spp.

La presencia de SARM, *S. aureus* MR, y el serotipo ST398 en la cadena productiva de la carne indica que existe un riesgo de transmisión a las personas. La similitud genética encontrada entre las cepas de origen porcino (animales y carne) sugiere una posible contaminación de la carne durante la faena. Cepas de *S. aureus* que presentan los genes *mecA* y PVL se encuentraron en pacientes con infecciones invasivas. La proporción de portadores sanos de *S. aureus* es baja, sin presentar los genes *mecA* y PVL, lo cual representa un riesgo menor de transmisión a la comunidad. El método de PCR en tiempo real puede recomendarse como una técnica rápida para la detección de *S. aureus* y el gen *mecA*, con la posterior confirmación de SARM a través del método estándar de cultivo.

Los resultados de este trabajo sugieren que debe implementarse una vigilancia efectiva en la cadena productiva de la carne con el fin de monitorear SARM y *S. aureus* MR y establecer acciones para disminuir la propagación de cepas resistentes a antibióticos.

Palabras claves: *Staphylococcus aureus* resistente a meticilina (SARM), multirresistente (MR), *mecA*, Leucocidina de Panton- Valentine (PVL), carne cruda, animales, productos cárnicos, aislados clínicos, personas sanas.

GENERAL ABSTRACT

The emergence and the spread of antimicrobial-resistant (AR) pathogens has increased the public health concern worldwide. The emergence of AR pathogens in food-producing animals has been asociated with the use of antibiotics in animal production in prophylaxis, therapy, and growth promotion. The exposure to sub-therapy doses of antimicrobial agents has created a reservoir of AR pathogens in animals with the risk of transmission to humans through the meat production chain. One of the pathogens that rapidly develop multidrug resistance (MDR) is *Staphylococcus aureus*. Particularly, methicillin-resistant *S. aureus* (MRSA) strains has become an important cause of health care-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) infections. The ability of *S. aureus* to colonize the nares and the skin of humans and animals, and the detection of MRSA and MDR strains in meat-producing animals and retail meat, have increased the probability of contamination of food in different steps of the food production chain. Methicillin resistance is attributed to the penicillin-binding protein 2a (PBP2a) encoded in the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*). This protein gives MRSA strains a reduced affinity for β-lactam antibiotics. CA-MRSA strains are more likely to encode the Panton–Valentine leukocidin (PVL) exotoxin, which is a virulence factor related to skin infections and tissue necrosis. Therefore, since the impact of those *S. aureus* strains in human health it is important to evaluate whether food could be a source of contamination that facilitate the spread of infections.

The aim of this study was to stablish the prevalence and molecular types of *S. aureus* and MRSA from meat-producing animals, retail raw meat, deli meat, and humans, determining the phylogenetic relationship between the strains.

A two-step selective enrichment followed by a culture method 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 PCR (mPCR) in order to identify the genes 16S rRNA (identification of *S. aureus*), *mecA* (detection of MRSA) and PVL-encoding genes (virulence factor associated to CA-MRSA). Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used for molecular typing of *S. aureus* strains.

Antimicrobial susceptibility testing was carried out using the broth microdilution method. In addition, *S. aureus* from nasal swabs obtained from 550 healthy subjects was isolated and identified by culture method and biochemical testing. A total of 108 MRSA isolates recovered from hospital patients were also examined by mPCR to confirm *S. aureus* and to detect *S. aureus* harboring the *mecA* and PVL genes. A multiplex real-time PCR assay was developed in order to decrease the time of detection in animal and meat samples and increase the sensibility of detection of *S. aureus* and the *mecA* and PVL genes. This procedure was carried out after the primary and the secondary enrichments that preceding the culture method. The results were compared with those obtained by the culture method which includes the two-step selective enrichment in order to isolate *S. aureus*. A total of 234 samples were examined by both methods (77 animal nasal swabs, 112 retail raw meat, and 45 deli meat).

The overall prevalence of *S. aureus* determined by the culture method with two-step selective enrichment was 34.7% in animals, with the highest rate found in pigs (50.0%) and sheep (40.6%) ($P<0.05$). In raw meat the prevalence was 47.6%, with the highest rate in chicken (67.6%) and pork (49.3%) ($P<0.05$). In deli meat the prevalence was 13.0% (Figure 2). Five pork samples (7.0%) were positive for *mecA* gene, of which three were serotype (ST) ST398 and two were ST5. All these strains exhibited penicillin resistance and four were MDR strains. The PVL genes were not detected in any sample by mPCR. The most common serotypes in sheep were ST398 and ST133, in pigs and pork meat both ST398 and ST9, and in chicken ST5. A genetic similarity between *S. aureus* strains ST9 isolated from pigs and pork meat was obtained. Most susceptible *S. aureus* strains to antimicrobial agents were ST5 isolated from chicken. The MDR isolates were found in pigs, pork meat, and sheep.

The prevalence of nasal carriage of *S. aureus* in healthy people was 7.6%. None of these strains were *mecA*- nor PVL-positive. A total of 105 MRSA strains (97.2%) obtained from hospital patients harbored the *mecA* gene and 11 (10.2%) PVL genes.

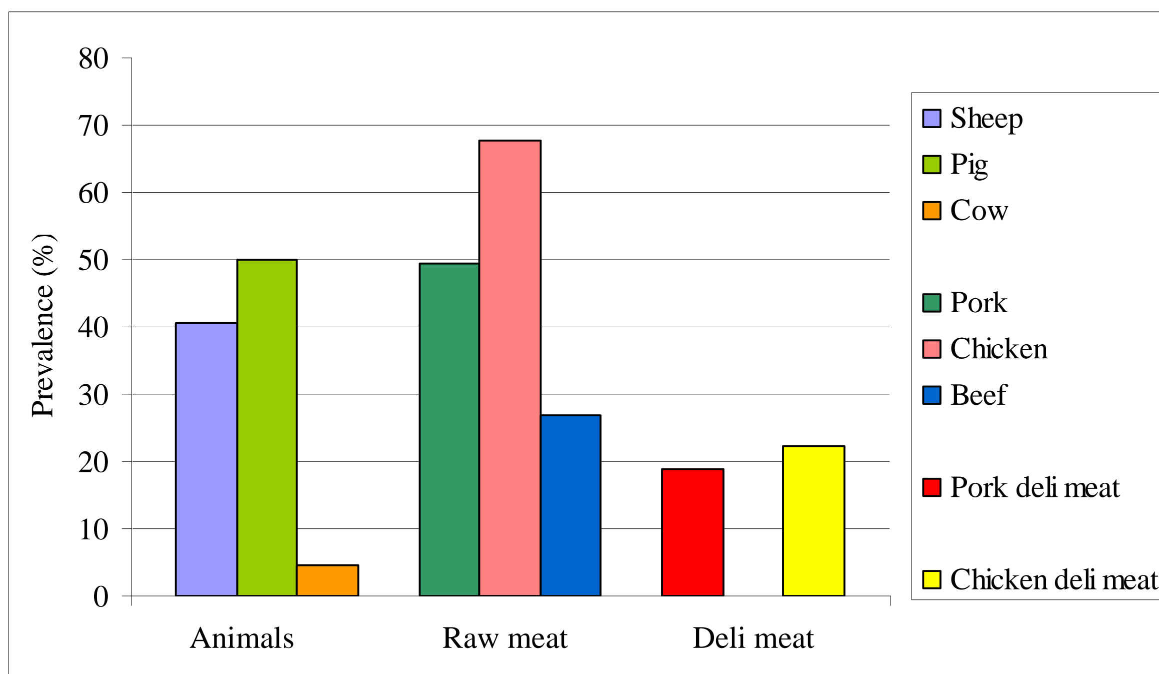

Figure 2. Prevalence of *Staphylococcus aureus* in animals, raw meat, and deli meat.

The application of real-time PCR assay on samples following primary and secondary enrichments detected *S. aureus* in 111/234 and 120/234 samples, respectively. These detection levels were higher than recovery obtained by the culture method, which was 95/234. Total agreement with the culture method for detection of *S. aureus* using real-time PCR was 83.9-97.8% (*kappa*=0.68-0.88 [from substantial to almost perfect agreement]), and 68.9-88.3% (*kappa*=0.29-0.77 [from fair to substantial agreement]) for primary and secondary enrichments, respectively. For detection of the *mecA* gen, total agreement was 91.1-98.7% and 86.7-98.7% (*kappa*=0-0.49 [from no agreement beyond that expected by chance to moderate agreement]) for primary and secondary enrichment samples, respectively. The real-time PCR assay detected *mecA* gene in some samples that were negative for *S. aureus*, but positive for *Staphylococcus* spp.

The presence of MRSA, MDR *S. aureus*, and the serotype ST398 in the meat production chain indicates that there is a risk of transmission to humans. The genetic similarity between strains of porcine origin (animals and meat) suggests the possible contamination of meat during slaughtering. *S. aureus* harboring *mecA* and PVL genes is present in patients affected by invasive infections. The rate of nasal carriage of *S. aureus* in healthy humans

seems to be low, without the presence of *mecA* and PVL genes, which represents a low risk of transmission among the community. The real-time PCR assay may be recommended as a rapid method for the detection of *S. aureus* and the *mecA* gen, with further confirmation of MRSA using the standard culture method.

The results of this study suggest that an effective surveillance should be implemented in the meat production chain in order to monitor MRSA and MDR *S. aureus*, and to take actions to decrease the spread of AR strains.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA), multidrug resistant (MDR), *mecA*, Panton-Valentine Leukocidin (PVL), retail raw meat, animals, deli meat, clinical isolates, healthy humans.

I. INTRODUCCIÓN GENERAL

La emergencia y propagación de patógenos resistentes a múltiples agentes antimicrobianos ha aumentado la preocupación en materia de salud a nivel mundial. La emergencia de cepas bacterianas resistentes a antibióticos en animales se relaciona con la utilización de antibióticos en la etapa productiva (de Neeling et al., 2007). Numerosos agentes antimicrobianos se suministran en la dieta de los animales con fines preventivos y de promoción del crecimiento, lo cual expone a un gran número de animales a concentraciones sub-terapeúticas de antibióticos en forma frecuente (DuPont y Steele, 1987; Franco et al., 1990), aumentando la probabilidad de generar bacterias resistentes a antibióticos. Esto puede tener como consecuencia que los genes de resistencia sean traspasados a bacterias presentes en humanos, lo que representa un riesgo latente para la pérdida de eficacia de los antibióticos utilizados en salud humana (Smith et al., 2002).

Existen evidencias sobre la contaminación de la carne con bacterias resistentes a múltiples agentes antimicrobianos, como *Salmonella*, *Campylobacter*, *Enterococcus* y *Escherichia coli* (FDA, 2010). En animales y en carne se encontraron cepas de *Staphylococcus aureus* resistente a meticilina (SARM) y *S. aureus* multirresistente (MR) (Waters et al., 2011; de Neeling et al., 2007). Sin embargo, no se tiene suficiente información sobre la prevalencia de SARM y *S. aureus* MR en alimentos de origen animal.

En humanos, *S. aureus* puede ocasionar una amplia gama de enfermedades como intoxicación alimentaria, neumonía, infecciones de heridas e infecciones nosocomiales (hospitalarias) (Tiemersma et al., 2004; Kennedy et al., 2008). Esta bacteria es un patógeno oportunista que se propaga a través del contacto directo de animales o personas con heridas infectadas o con otros procesos infecciosos. La transmisión también puede ocurrir desde personas o animales colonizados por esta bacteria que son portadores asintomáticos (CFSPH, 2011).

Staphylococcus aureus y SARM pueden colonizar las fosas nasales y la piel de los animales (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), lo cual aumenta el riesgo de contaminación de las canales durante la faena de animales de abasto (de Boer et al., 2009).

En Estados Unidos se estima que la prevalencia de colonización nasal con *S. aureus* en humanos es de aproximadamente del 29% y con SARM de. 1,5% (Gorwitz et al., 2008). Por lo tanto, las personas también constituyen una fuente de contaminación durante la manipulación de los alimentos. De esta forma, los alimentos contaminados por esta vía se transforman en vehículos para diseminar este patógeno, siendo los de mayor riesgo aquellos sometidos a una cocción inadecuada (CFSPH, 2011) y aquellos que no requieren cocción para su consumo.

En investigaciones anteriores se determinó la prevalencia y se tipificaron molecularmente diferentes cepas de *S. aureus* y SARM en muestras de animales y carne (Waters et al., 2011; de Neeling et al., 2007) y, en otras investigaciones en muestras de humanos (Tiemersma et al., 2004; Kennedy et al., 2008), siendo difícil establecer la relación o similitud genética entre ellas. Este estudio pretende establecer la prevalencia de *S. aureus*, cepas sensibles y resistentes a la meticilina, en animales, carne, productos cárnicos y humanos y determinar la relación filogenética de las cepas aisladas.

1. Características de *Staphylococcus aureus*

Inicialmente, el género *Staphylococcus* fue clasificado dentro de la familia *Micrococacea*e. Sin embargo, estudios recientes de homología genética demostraron que los géneros *Staphylococcus* y *Micrococcus* tienen poca relación. Por esta razón, *Staphylococcu*s se incluyó en la familia *Staphylococcaceae*, dentro del orden *Bacillales* (Euzéby, 1997). El nombre del género -*Staphylococcus*- proviene del griego *staphylé* que significa racimo de uvas, ya que presentan forma esférica (cocos), con un diámetro de 0,5 a 1,5 µm y se agrupan de forma irregular. Éstas son bacterias gram positivas, inmóviles, no forman esporas y, generalmente, no poseen cápsula y son anaerobias facultativas. Se diferencian de los géneros *Streptococcus* y *Enterococcus* por producir la enzima catalasa, la cual hidroliza el peróxido de hidrógeno (H_2O_2) en oxígeno (O_2) y agua (H_2O). Presentan metabolismo oxidativo y fermentativo de la glucosa, a diferencia del género *Micrococcus*, el cual no la fermenta (de Cueto y Pascual, 2009).

En un medio de cultivo no selectivo *S. aureus* presenta colonias lisas, solelevadas, brillantes, de consistencia cremosa, de color amarillo o dorado, debido a la producción de un pigmento carotenoide. Esta especie es resistente al calor y a la desecación y tiene la capacidad

de crecer en medios de alta salinidad (7,5% de NaCl). En medios de cultivo que contienen sangre, la mayoría de las cepas produce lisis de los glóbulos rojos por acción de la β-hemolisina (β-hemólisis), lo cual se manifiesta con un halo claro alrededor de las colonias. Una de las características que diferencia a *S. aureus* de otras especies, salvo excepciones, es la producción de la enzima coagulasa, que transforma el fibrinógeno en fibrina coagulando el plasma sanguíneo (Lowy, 1998; de Cueto y Pascual, 2009). Adicionalmente, *S. aureus* produce una ADNasa termoestable que rompe los enlaces fosfodiéster del ADN. Esta propiedad sirve como método de identificación, dado que al someter a *S. aureus* a altas temperaturas y luego incubar con ADN, se observará la degración de ADN (de Cueto y Pascual, 2009).

Staphylococcus aureus presenta diferentes factores de virulencia. Posee componentes microbianos de superficie que reconocen moléculas adhesivas de la matriz (MSCRAMM, del inglés *microbial surface components recognizing adhesive matrix molecules*), las cuales median la adherencia a los tejidos, tales como el factor de agregación (clumping factor), proteínas de unión al fibrinógeno, fibronectina y sialoproteína ósea (Lowy, 1998; de Cueto y Pascual, 2009). Además, esta bacteria produce un polisacárido de adherencia intracelular que permite su persistencia a través de la formación de una biocapa bacteriana que le confiere protección (de Cueto y Pascual, 2009). Para evadir los mecanismos de defensa del huésped, *S. aureus* presenta la proteína A, proteína de adherencia extracelular y citotoxinas (Leucocidina de Panton-Valentine [PVL], α-toxina). Adicionalmente, sintetiza lipasas, hialuronidasas y proteasas, las que destruyen los tejidos, facilitando la propagación de la infección. Otros factores de virulencia son los asociados a las intoxicaciones alimentarias y shock tóxicos: enterotoxinas, toxina del síndrome del shock tóxico 1, toxinas exfoliativas A y B y α-toxina (Lowy, 1998; de Cueto y Pascual, 2009). Todos estos factores de virulencia presentes en *S. aureus* promueven la colonización e invasión, ocasionando un daño severo al hospedero.

2. Mecanismos de resistencia a meticilina

Existen muchos agentes con actividad antiestafilocócica, sin embargo, *S. aureus* ha desarrollado mecanismos para neutralizarlos, encontrándose cepas resistentes a múltiples agentes antimicrobianos (McDougal et al., 2003; Aydin et al., 2011; Waters et al., 2011).

En Europa, a principios de la década de los 60s, poco después de la introducción de la meticilina (penicilina semisintética resistente a penicilinasa) surgió *S. aureus* resistente a meticilina (SARM), asociado a infecciones originadas en instalaciones hospitalarias. A fines de la década de los 90s, emergieron en todo el mundo cepas de SARM asociados a infecciones originadas en la comunidad (Lowy, 1998, 2003; Deurenberg y Stobberingh, 2008). En consecuencia, la propagación global de SARM ha generado preocupación mundial (Voss y Doebbling, 1995), debido a la aparición creciente de infecciones nosocomiales (Tiemersma et al., 2004), comunitarias (Kennedy et al., 2008) y en animales (Golding et al., 2010).

La meticilina es un antibiótico β-lactámico, al igual que la penicilina G, oxacilina, ampicilina, amoxicilina y cefalosporinas, entre otros. Estos antibióticos inhiben la síntesis de la pared celular de bacterias gram positiva, mediante la inhibición de la última etapa de la síntesis del peptidoglicano, correspondiente a la unión del ácido N-acetilmurámico a la pared celular, lo que se conoce también como transpeptidación. La transpeptidación está catalizada por transpeptidasas y carboxipeptidasas, llamadas también proteínas de unión a penicilina (PBPs) por su capacidad para fijar penicilina en su sitio activo. El anillo β-lactámico se une de forma covalente a una serina presente en el sitio activo de las PBPs, inactivando la transpeptidación. Así, la pared celular queda debilitada y puede romperse por la presión osmótica intracelular. Existen PBPs que inhiben enzimas autolíticas de la pared bacteriana (autolisinas), por lo cual al unirse la penicilina al sitio de fijación de las PBPs, no se inhiben las autolisinas produciendo la lisis celular (Marín y Gudiol, 2003; Romero, 2007).

Uno de los mecanismos de resistencia a antibióticos β-lactámicos corresponde a la acción de la enzima β-lactamasa, la cual confiere resistencia a la penicilina, debido a que hidroliza el anillo β-lactámico, inactivando al antibiótico. Esta enzima está codificada en el gen *blaZ*, que se encuentra en un transposón ubicado en un plásmido junto con otros genes asociados a resistencia antimicrobiana (Lowy, 2003).

La resistencia a la meticilina presente en SARM confiere resistencia a todas las penicilinas resistentes a penicilinasa y cefalosporinas (Lowy, 1998). Esto se atribuye a la proteína de unión a la penicilina 2a (PBP2a, penicillin-binding protein 2a), que posee baja afinidad por los antibióticos β-lactámicos (Hartman y Tomasz, 1981; Lim y Strynadka, 2002). La proteína PBP2a se diferencia de otras PBPs en que su sitio activo no permite la fijación de

antibióticos β-lactámicos, posibilitando el desarrollo de la reacción de transpeptidación. De esta manera, al formarse la pared celular, se favorece la supervivencia de los estafilococos expuestos a altas concentraciones de estos agentes antimicrobianos (Lim y Strynadka, 2002). La proteína PBP2a está codificada por el gen *mecA*, el cual se encuentra en un elemento genético móvil denominado cassette cromosómico estafilocócico mec (SCC*mec*) (Hartman y Tomasz, 1981). La expresión del gen *mecA* es regulada por dos proteínas: MecI, represor codificado por el gen *mecI*; y MecR1, proteína transductora de señal codificada por el gen *mecR1*. En ausencia de antibióticos β-lactámicos, la proteína MecI se une al operador, reprimiendo la transcripción del gen *mecA*. La fijación de antibióticos β-lactámicos a la proteína MecR1, provoca la liberación de un fragmento con actividad proteasa de esta proteína, el cual divide al represor MecI en fragmentos inactivos, permitiendo la transcripción del gen *mecA* y la posterior síntesis de PBP2a (Lowy, 2003).

3. Tipificación molecular de *Staphylococcus aureus* y SARM

La tipificación de las cepas de *S. aureus* no está completamente estandarizada y a través de los años se han utilizado una gran variedad de métodos (Tenover et al., 1994). Dentro de las técnicas moleculares que se utilizan actualmente para tipificar las cepas de SARM está: la electroforesis en gel de campo pulsado (PFGE), basado en la macro-restricción del ADN genómico; la tipificación de secuencias multilocus (MLST), basada en el perfil alélico de siete genes conservados; y la tipificación de *spa*, basado la secuenciación de la región X del gen que codifica la proteína A presente en *S. aureus* (McDougal et al., 2003). Por esta razón, una misma cepa puede recibir diferentes nombres (CFSPH, 2011). Los Centros para el Control y la Prevención de Enfermedades (CDC, del inglés *Centers for Disease Control and Prevention*) establecieron un sistema de nomenclatura para *S. aureus* y SARM comunes en Estados Unidos, basado en los patrones de macro-restricción obtenidos a través de PFGE, identificando ocho tipos, desde USA100 hasta USA800 (McDougal et al., 2003). La nomenclatura utilizada para MLST corresponde a serotipos (ST) seguido de un número (ej. ST398), mientras que para tipificación de *spa* se utiliza "t" seguido de un número (ej. t011) (Cuny et al., 2010). Generalmente, PFGE y MLST clasifican las cepas en clusters similares (Catry et al., 2010), los cuales pueden contener diferentes tipos *spa*. Una de las desventajas de la tipificación de *spa* es que líneas clonales no relacionadas pueden tener tipos *spa* similares (Van den Broek IV et al.,

2009; Golding et al., 2008). Esto se debe a que la tipificación de *spa* se centra en una pequeña fracción del genoma y, una recombinación, podría resultar en discrepancias con los clusters obtenidos a través de MLST y PFGE (Golding et al., 2008). Se ha demostrado que el método PFGE tiene mayor poder de discriminación que MLST y la tipificación de *spa*. Sin embargo, estas técnicas pueden utilizarse para determinar cambios mayores que pueden ocurrir en las líneas clonales a través del tiempo (McDougal et al., 2003). Para lograr una mayor precisión en la tipificación de las cepas se recomienda la combinación de dos métodos (Tenover et al., 1994).

Con la utilización de PFGE se determinó que las cepas de SARM causantes de infecciones adquiridas en la comunidad (USA300 y USA400) son diferentes a las cepas nosocomiales (USA100 y USA200) (McDougal et al., 2003). Vandenesch et al. (2003) determinaron que las cepas de SARM comunitario provenientes de tres continentes comparten dos genes, el cassette cromosómico SCC*mec* tipo IV y el locus PVL, que contiene los genes productores de la toxina PVL. Los genes PVL están localizados en un bacteriófago que infecta a *S. aureus*, mientras que la distribución de otros genes de toxinas es específica de las cepas de cada continente. La mayoría de las cepas de SARM comunitario presentan el locus PVL (Baba et al., 2002; Dufour et al., 2002), factor de virulencia asociado a infecciones de piel, neumonía y necrosis de tejidos (Ebert et al., 2009).

A través del método MLST se ha podido determinar la presencia de algunos tipos de secuencias asociadas a SARM hospitalario, como ST5, ST8, ST22, ST36, ST45, entre otros (Deurenberg et al., 2007); mientras que ST30 y ST80 han sido asociados a SARM comunitario (Stenhem et al., 2010) y ST398 a SARM en animales, específicamente en cerdos (Lewis et al., 2008; van Belkum et al., 2008; Krziwanek et al., 2009). El serotipo ST398, inicialmente asociado a cerdos, ha sido encontrado en personas, la mayoría productores de cerdos (van Belkum et al., 2008; Krziwanek et al., 2009; Pan et al., 2009; Golding et al., 2010). Además, recientemente se asociaron infecciones a SARM ST398 en humanos en contacto con vacas lecheras con mastitis sub-clínica (Soavi et al., 2010). No obstante, en Suecia, se reportaron dos casos de ST398 t038 en pacientes que no tuvieron contacto con animales (Welinder-Olsson et al., 2008). Esto sugiere la propagación de estas cepas en el ambiente, facilitando la colonización de personas que no están involucradas con la producción animal, generando una creciente preocupación en salud pública (Gibbs et al., 2006). *Staphylococcus aureus* resistente

a meticilina ST398 no es tipificable mediante PFGE, debido a que su ADN no puede ser digerido por la enzima de restricción *Sma*I, ya que posee una enzima que metila la secuencia de reconocimiento de *Sma*I (Bens et al., 2006). Análisis comparativos de la huella genética de cepas de SARM ST398 demostraron que existe diversidad molecular y geográfica (Golding et al., 2010). Además, existe evidencia que demuestra la aparición de cepas de SARM diferentes a ST398, como ST9 t899, asociado también a la producción de cerdos (Guardabassi et al., 2009). Por lo tanto, la emergencia de nuevas cepas de SARM en cerdos resalta la importancia de establecer estrategias de vigilancia permanente, determinando el riesgo de transmisión a las personas relacionadas con producción porcina.

4. Prevalencia de *Staphylococcus aureus* y SARM

El método para aislar *S. aureus* y SARM no está estandarizado. Por lo tanto, la utilización de diferentes métodos puede afectar la sensibilidad de la detección y, por consiguiente, los resultados de prevalencia. Algunos análisis han incluido solamente cultivo en placa utilizando agar manitol sal (MSA) con 2 µg/mL de oxacilina (Weese et al., 2006) o agar Baird Parker (BP) (Aydin et al., 2011). Otros estudios han incorporado etapas de enriquecimiento previo al cultivo en placa. Wertheim et al. (2001) desarrollaron un caldo selectivo con rojo fenol, manitol, aztreonam y ceftizoxima ($PHMB^{+}$), lo cual aumentó al doble la sensibilidad de detección de SARM. Broens et al. (2011) utilizaron un protocolo de dos etapas de enriquecimiento, una con caldo Mueller-Hinton con 6,5% de NaCl (MHB+6,5%NaCl) y la otra con $PHMB^{+}$, seguido de cultivo en agar cromogénico selectivo para SARM. Otros autores han utilizado enriquecimiento en caldo con 7,5% de NaCl, 1% de manitol y 2,5% de extracto de levadura seguido de un medio cromogénico (Zhang et al., 2011); caldo tripticasa soya suplementado con 10% de NaCl y 1% de piruvato de sodio seguido de cultivo en agar BP (Pu et al., 2009; Pu et al., 2011); $PHMB^{+}$ seguido de cultivo en agar con sangre ovina y dos agares selectivos (Tenhagen et al., 2009). Estos distintos métodos sugieren que para aumentar la sensibilidad de detección de *S. aureus* y SARM se debería incluir una etapa de enriquecimiento seguida de un cultivo selectivo.

La mayoría de los animales pueden ser colonizados con *S. aureus*, encontrándose en las fosas nasales y la piel (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). Por esta razón, durante la

faena existe el riesgo de contaminación de las canales y la carne con *S. aureus* y SARM (de Boer et al., 2009). Recientemente, se aislaron cepas de SARM en cerdos, vacunos y pollos (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). En Holanda de Neeling et al. (2007) encontraron una alta prevalencia de SARM ST398 (39%) en cerdos y una alta tasa de resistencia a diferentes antibióticos (tetraciclina, eritromicina, clindamicina, kanamicina, gentamicina, tobramicina) sugiriendo, además, la existencia de contaminación entre los animales en los corrales de las plantas faenadoras. En un estudio realizado en Hong Kong se determinó una prevalencia de SARM en cerdos menor (16%) a la reportada en el estudio anterior (Guardabassi et al., 2009), lo que podría deberse al tamaño menor de la muestra analizada y al método utilizado en la detección. Dado que los cerdos constituyen una posible fuente de infección de SARM se requiere estudiar la epidemiología de esta zoonosis emergente, determinando la frecuencia de transmisión de SARM desde animales a humanos, y la transmisión persona-persona (Lewis et al., 2008).

En los últimos años se han aislado cepas de SARM en carne de cerdo, pollo, vacuno, pavo y cordero. Hanson et al. (2011) analizaron diferentes tipos de carne de supermercados del Estado de Iowa, Estados Unidos, encontrando sólo 2 muestras de carne de cerdo contaminadas con cepas de SARM, con una prevalencia total de alrededor de 1%. En Estados Unidos, un estudio realizado en el Estado de Louisiana reveló contaminación con *S. aureus* en 45,6% de las muestras de carne de cerdo y en 20% de las muestras de carne de vacuno, de los cuales el 5,6% y el 3,3% correspondió a cepas de SARM en carne de cerdo y vacuno, respectivamente (Pu et al., 2009). de Boer et al. (2009) encontraron una mayor porporción de carne comercializada en supermercados de Holanda contaminada con SARM, principalmente ST398, de alrededor de un 35% en carne de pavo, 16% en carne de pollo, 11% en carne de cerdo, 10% en carne de vacuno y 6% en carne de cordero. Estos últimos valores de prevalencia de SARM en carne sugieren que el método utilizado en aquél estudio (dos etapas de enriquecimiento selectivo + cultivo selectivo) permitió una mayor sensibilidad de detección de SARM. Finalmente, la detección de SARM en carne ha aumentado la preocupación en materia de inocuidad alimentaria con respecto a la cadena de producción de carne y ha generado la urgencia de contar con sistemas de vigilancia y coordinación entre diferentes laboratorios de análisis.

En Estados Unidos, una de cada seis personas contrae una enfermedad transmitida por alimentos cada año, con un total de 48 millones de personas afectadas. De los agentes causantes, *S. aureus* es uno de los cinco principales, con un total de 240 mil casos, correspondientes a una enfermedad gastrointestinal causada por el consumo de toxinas producidas por *S. aureus* (CDC, 2012). Por otro lado, las cepas de SARM están asociadas principalmente a infecciones de la piel en la comunidad, y los casos más graves se asocian a pacientes hospitalizados, afectados por infecciones sanguíneas, quirúrgicas, o neumonía (CDC, 2011). Aproximadamente el 29% de la población en Estados Unidos es portadora de *S. aureus* y el 1,5% de SARM en las fosas nasales (Gorwitz et al., 2008). Del total de 478.000 casos de infecciones por *S. aureus* que resultaron en hospitalizaciones en el año 2005, aproximadamente el 50% se asoció a SARM, y del total de 11.406 muertes asociadas a *S. aureus*, 6.639 correspondieron a infecciones por SARM (Klein et al., 2007).

En el Estado de Dakota del Norte se cuenta con información epidemiológica acerca de las infecciones causadas por SARM en humanos. Sin embargo, no existe información acerca de la prevalencia de cepas de SARM en animales o carne. En el año 2011, se registraron 13 casos de infecciones asociadas a SARM por cada 100.000 habitantes. Estas infecciones aumentaron desde el año 2001 hasta el año 2006, manteniéndose actualmente sin variación (North Dakota Department of Healt, Disease Control, 2011).

Las infecciones causadas por microorganismos resistentes a antibióticos tienen un costo económico considerablemente alto. En promedio, el costo de hospitalización por infecciones de SARM es de aproximadamente US$ 14.000, comparado con US$ 7.600 de hospitalización por infecciones no-SARM. Por lo tanto, el costo total de las hospitalizaciones por infecciones de SARM en Estados Unidos asciende a más de 3 billones de dólares al año. (Elixhauser y Steiner, 2007). Lo anterior no incluye las pérdidas que se generan por ausentismo laboral, incapacidad y mortalidad.

La prevención y control de infecciones de SARM en Estados Unidos está dirigida por el CDC, que proporciona información específica para cada grupo de la población y estrategias para el tratamiento de infecciones de SARM en la comunidad. La disminución de infecciones de SARM en hospitales y en la comunidad es de alta prioridad para CDC. Es así como se establecieron proyectos de vigilancia a corto y largo plazo con la colaboración de los

departamentos de salud, hospitales, centros médicos, entre otros (CDC, 2011). Sin embargo, se necesitan más estudios para poder caracterizar genéticamente las cepas de SARM y determinar las rutas de transmisión en animales y humanos, lo que facilitará el desarrollo de acciones preventivas para disminuir su propagación.

5. Hipótesis y objetivos

Hipótesis

Staphylococcus aureus, cepas sensibles y resistentes a la meticilina, están presentes en animales, carne, y humanos, existiendo relación filogenética entre las cepas.

Objetivo general

Establecer la prevalencia y los tipos moleculares de *S. aureus* y SARM en animales de abasto, carne cruda, productos cárnicos y humanos, determinando la relación filogenética entre las cepas.

Objetivos específicos

- Determinar la presencia de cepas de *S. aureus* en animales, carne, productos cárnicos y humanos.
- Determinar la prevalencia de *S. aureus* y SARM en animales, carne, productos cárnicos y humanos.
- Determinar características moleculares de las cepas de *S. aureus* y SARM a través de las técnicas de PCR múltiple, electroforesis en gel de campo pulsado y análisis de secuencias multilocus.
- Determinar el perfil de resistencia antimicrobiana de las cepas de *S. aureus* y SARM.
- Comparar la técnica de PCR en tiempo real con el método de cultivo y PCR convencional, utilizando dos etapas de enriquecimiento selectivo, para la detección de *S. aureus* y SARM en muestras de animales, carne y productos cárnicos.

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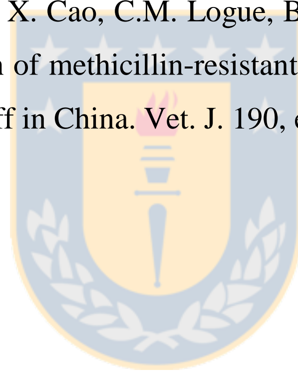

II. DESARROLLO

1. Molecular typing of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolated from animals and retail meat in North Dakota, United States

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Abstract

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, and the highest prevalence observed in pigs (50.0%) and sheep (40.6%) ($p<0.05$); 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

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reaction. The most common clones in sheep were ST398 and ST133, in pigs and pork meat both ST398 and ST9, and in chicken ST5. Most susceptible *S. aureus* strains were ST5 isolated from chicken. The MDR isolates were found in pigs, pork, and sheep. The presence of MRSA, MDR, and the subtype ST398 in the meat production chain and the genetic similarity between strains of porcine origin (meat and animals) suggest the possible contamination of meat during slaughtering and its potential transmission to humans.

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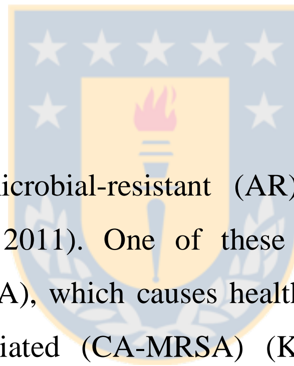

1.1 Introduction

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 (Tiemersma et al., 2004), community-associated (CA-MRSA) (Kennedy et al., 2008), and livestock-associated 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). 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. suggest that a combination of two methods may provide more precision in epidemiological studies (Tenover et al., 1994).

It has been demonstrated that MRSA strains causing CA-MRSA infections (USA300 and USA400) are different from those causing health care-associated MRSA infections (USA100 and USA200) (McDougal et al., 2003). The sequence type ST398 has been associated with livestock-associated 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.

1.2 Materials and Methods

1.2.1 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 ND Veterinary Diagnostic Lab (North Dakota State University, Fargo, ND).

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.

1.2.2 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 18-20 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 18-20 h at 37°C.

Nasal swabs were placed directly in 9 mL MHB+6.5% NaCl and incubated for 18-20 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 18-20 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.

1.2.3 Multiplex polymerase chain reaction (PCR)

All *S. aureus* strains were recovered from frozen stock to TSA plates and incubated at 37°C for 18-24 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.

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 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 35591) were included on each gel. Bands were visualized using an Alpha Innotech UV imager (FluorChem™).

1.2.4 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 18-24 h. A single colony was inoculated into a second TSA plate and incubated at 37°C for 18-24 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 0.9-1.1 in a spectrophotometer (Smart Spec™ 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 an initial switch time of 5 s, a final switch time of 40 s, and a 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%.

1.2.5 Multilocus sequence typing (MLST)

Briefly, *S. aureus* isolates were struck to TSA plates and incubated at 37°C for 18-24 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 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). 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.

1.2.6 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).

1.2.7 Statistical analysis

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

1.3 Results

Table 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 meats (13.0%, n=6). MRSA was detected solely in meat (five pork samples), representing a low prevalence ($p<5\%$). The PVL gene was not detected in any sample.

Most of the *S. 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 3).

A total of 47.7% (n=41) of the penicillin-resistant *S. aureus* strains exhibited MICs between 0.5 and 1 µg/mL. However, MRSA strains had higher MICs for penicillin (1- >16 µg/mL) (Table 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 5).

Figure 1 shows a dendrogram displaying the macrorestriction patterns of *S. aureus* strains and the sequence type (ST). The largest cluster (cluster 4) contained *S. aureus* of porcine origin (animals and meat), all of which were ST9. *S. 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 animals (sheep and pigs) and from pork meat (data not shown).

1.4 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). 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.

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 cattle (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 meats 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 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).

Other authors have also determined a higher occurrence of resistance to penicillin, tetracycline, and erythromycin in *S. aureus* strains isolated from retail meats 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).

AR *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). 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 4).

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 ST398 was found, which may indicate the potential risk for humans to acquire this emerging sequence type that has potential for causing infection.

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. MDR 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 MDR 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).

1.5 Conclusion

The presence of MDR *S. aureus*, and subtype ST398 in animals and meat, and MRSA in retail meat, and 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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Disclosure Statement

No competing financial interests exist.

1.6 References

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Table 1 (Desarrollo 1). Nucleotide sequence of the primers used in multiplex polymerase chain reaction for detection of 16S rRNA, *mecA*, and Panton-Valentine leukocidin genes (McClure *et al*., 2006); and multilocus sequence typing analysis for detection of *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* genes (Enright *et al*., 2000).

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 AA3'	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'	

Table 2 (Desarrollo 1). 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*	*No. of samples positive for S. aureus (%)*	*No. of isolates with the specific gene (%)*		
			16S rRNA	*mecA*	*PVL*
Animal					
Sheep	64	26 (40.6)	26 (40.6)		
Pig	60	30 (50.0)	30 (50.0)		
Cow	43	2 (4.7)	2 (4.7)		
Total	167	58 (34.7)	58 (34.7)	0 (0.0)	0 (0.0)
Raw meat					
Pork	71	35 (49.3)	35 (49.3)	5 (7.0)	
Chicken	37	25 (67.6)	25 (67.6)		
Beef	37	10 (27.0)	10 (27.0)		
Total	145	69 (47.6)	69 (47.6)	5 (3.4)	0 (0.0)
Deli meat					
Ham	21	4 (19.0)	4 (19.0)		
Turkey	16	0 (0.0)	0 (0.0)		
Chicken	9	2 (22.2)	2 (22.2)		
Total	46	6 (13.0)	6 (13.0)	0 (0.0)	0 (0.0)

Table 3 (Desarrollo 1). Antimicrobial resistance of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from animals and retail meat.

Antimicrobial		*No. (%) of resistant all S. aureus isolates*			*No. (%) of resistant MRSA isolates*
Subclass	*Agent*	*Animal (n=58) (Sheep; Pig; Cow)*	*Raw meat (n=69) (Pork; Chicken; Beef)*	*Deli meat (n=6) (Ham;Turkey;Chicken)*	*Pork meat (n=5)*
Macrolides	Erythromycin	3 (5.2) (0; 3; 0)	28 (40.6) (25; 3; 0)	1 (16.7) (0; 0; 1)	4 (80.0)
Tetracyclines	Tetracycline	47 (81.0) (24; 23; 0)	29 (42.0) (27; 2; 0)		4 (80.0)
Fluoroquinolones	Ciprofloxacin		2 (2.9) (2; 0; 0)		
Phenicols	Chloramphenicol	3 (5.2) (0; 3; 0)	2 (2.9) (2; 0; 0)		
Penicillins	Penicillin	49 (84.5) (19; 30; 0)	35 (50.7) (27; 4; 4)	2 (33.3) (2; 0; 0)	5 (100.0)
Aminoglycosides	Gentamicin	1 (1.7) (1; 0; 0)	1 (1.4) (1; 0; 0)		
	Kanamycin		2 (2.9) (2; 0; 0)		1 (20.0)
	Streptomycin	6 (10.3) (1; 5; 0)			
Streptogramin	Quinupristin/dalfopristin		2 (2.9) (2; 0; 0)		
Lincosamides	Lincomycin	38 (65.5) (13; 24; 1)	29 (42.0) (26; 3; 0)	1 (16.7) (1; 0; 0)	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); nitrofutantoin (2-64); penicillin (0.25-16); kanamycın (128-1024); erythromycin (0.25-8); ciprofloxacin (0.12-4); vancomycin (0.25-32); lincomycin (1-8); gentamicin (128-1024). All isolates were susceptible to vancomycin, tigecycline, daptomycin, tylosin tartrate, nitrofurantoin and linezolid.

Table 4 (Desarrollo 1). Minimum inhibitory concentrations (MICs) of resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* isolates from animals and retail meat.

Antimicrobial Agent (breakpoints)	*Total*	*No. (%) of resistant S. aureus isolates with MIC (µg/mL) of*											
		0.5 - 1	*2*	*4*	*>4*	*8*	*>8*	*16*	*>16*	*32*	*>32*	*256*	*>256*
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)						

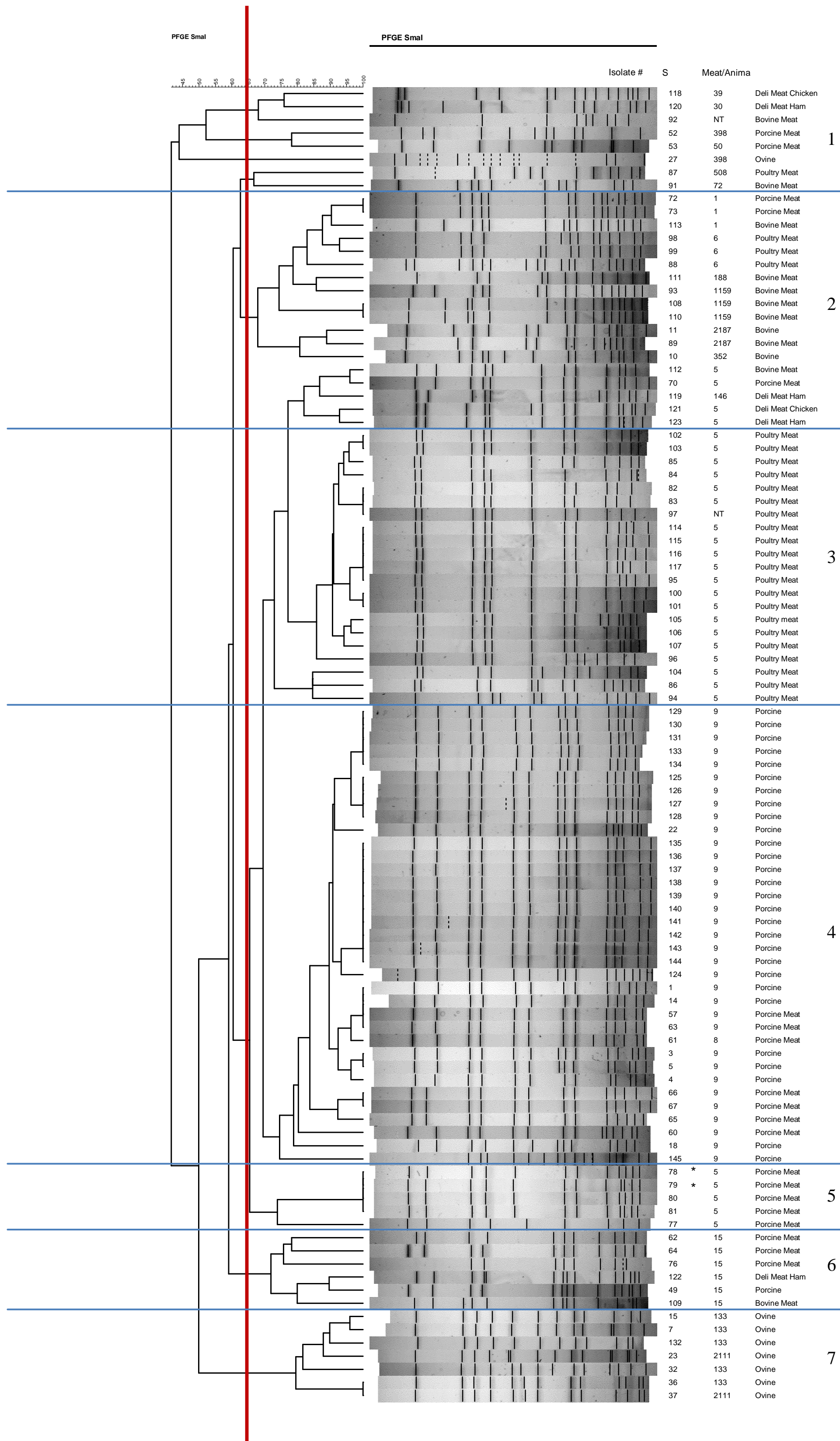
[a]Levels of MIC values against tested antibiotics (CLSI, 2009).
[b]Levels of MIC values against tested antibiotics (Jarløv et al., 1997).
[c]Levels of MIC values against tested antibiotics (Nemati et al., 2008).

Table 5 (Desarrollo 1). 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	No. (%) of all *S. aureus* isolates with the specific profile			No. (%) of MRSA isolates with the specific profile
		Animal (n=58)	Raw meat (n=69)	Deli meat (n=6)	Raw meat (n=5)
ERY-PEN-TET-LINC-CHL-GEN-CIP-QUI/DAL	8		1 (1.4)		
ERY-PEN-TET-LINC-CHL-CIP-QUI/DAL	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)		
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)	

CIP, ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; LINC, lincomycin; QUI/DAL, quinupristin/dalfopristin; PEN, penicillin; STR, streptomycin, TET, tetracycline.

Figure 1 (Desarrollo 1). Dendrogram showing the genetic relatedness of 100 *Staphylococcus aureus* isolates as determined by the analysis of pulsed-field gel electrophoresis (PFGE) profiles by the unweighted-pair group method with arithmetic averages with the sequence type (ST). The scale indicates levels of similarity within this set of isolates. Red line indicates the cutoff at 80%. Numbers represent the samples codes, followed on the right by the STs and the type of the sample. Clusters are separated by horizontal lines and labeled with correlative numbers. Asterisks indicate methicillin-resistant *Staphylococcus aureus* isolates determined by multiplex polymerase chain reaction targeting the *mecA* gene.


PFGE SmaI
PFGE SmaI
Isolate #
S
Meat/Anima
1
2
3
4
5
6
7

2. Multiplex real-time PCR for detection of *Staphylococcus aureus*, *mecA* and Panton-Valentine Leukocidin (PVL) genes from selective enrichments from animals and retail meat

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Abstract

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) and 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. The conventional culture/PCR method recovered 95/234 positive *S. aureus* samples. Application of real-time PCR on samples following primary and secondary enrichment detected *S. aureus* in 111/234 and 120/234 samples respectively. For detection of *S. aureus*, the *kappa* statistic was 0.68-0.88 (from substantial to almost perfect agreement) and 0.29-0.77 (from fair to substantial agreement) for primary and secondary enrichments, respectively, using real-time PCR. For detection of *mecA* gene, the *kappa* statistic was 0-0.49 (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 multi-drug 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 methicillin-resistant *S. aureus* (MRSA) using the standard culture method.

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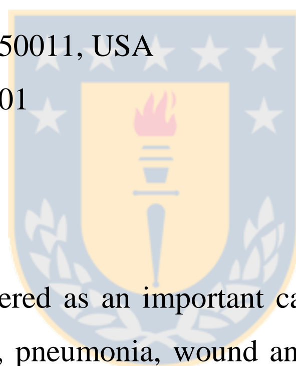

2.1 Introduction

Staphylococcus aureus is considered as an important cause of a wide variety of diseases in humans such as: food poisoning, pneumonia, wound and nosocomial infections [1,2]. There are many anti-staphylococcal agents; however, the bacterium has developed mechanisms to neutralize them such as the methicillin resistance mechanism [3]. Methicillin-resistant *S. aureus* (MRSA) is an increasing cause of health care-associated (HA-MRSA) [1], community-associated (CA-MRSA) [2], and livestock-associated (LA-MRSA) infections worldwide [4].

The altered penicillin-binding protein (PBP2a) is associated with methicillin resistance. This protein has a reduced affinity for β-lactam antibiotics [5,6], and is encoded by the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) [5]. 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 [7,8]. The PVL toxin has been related to life-threatening CA-MRSA infections and deaths, primarily severe skin infections and tissue necrosis [9].

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 [10]. An estimated 478,000 hospitalizations corresponded to *S. aureus* infections, of which 278,000 hospitalizations were attributed to MRSA infections in 2005 [11]. In addition, the carriage of MRSA in meat-producing animals [12-14] and the contamination of retail meat with MRSA [15-17] have increased the concern that food may serve as a vehicle to transmit MRSA to the human population [17].

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) [18] developed a selective media containing phenol red 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 3-7 days approximately [15,16,19]. 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. Detection using real-time PCR may decrease the time of analysis to 18 h after consecutive broth enrichment in clinical samples [20]; or <2 h in positive blood cultures [21,22]. 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 [23,24] and meat [15,25,26].

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, and there is a need to decrease the time of analysis, we analyzed samples obtained from animals and retail meats 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 obtained with the real-time PCR assay 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.

2.2 Materials and Methods

2.2.1 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 ND Veterinary Diagnostic Lab at North Dakota State University, Fargo, ND. Animal samples were collected during the period May 2010-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.

2.2.2 Culture method

Staphylococcus aureus were isolated by the two-step selective enrichment procedure according to the method described by de Boer *et al.* (2009) [15] 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 18-20 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] [18]), followed by incubation for 18-20 h at 37ºC. Nasal swabs from animals were placed directly in 9 mL MHB+6.5%NaCl and incubated for 18-20 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 18-20 h at 37ºC. Suspect *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.

2.2.3 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 18-24 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.

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 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) [27] 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 control (DNA extracted from MRSA ATCC 35591, positive for *mecA* and PVL genes) and an external negative control (DNase/RNase-free distilled water) were included with each run.

PCR amplicons (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) were included on each gel. Bands were visualized using an Alpha Innotech UV imager (FluorChem™).

2.2.4 Multiplex real-time PCR assay

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) [28]. Five microliters of DNA template extracted was used in the real-time iQ™ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), in a final volume of 20 µL per reaction.

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

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

Multilocus Sequence typing (MLST)

Briefly, *S. aureus* isolates were struck to TSA plates and incubated at 37°C for 18–24 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) [29]. The housekeeping genes: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*, were amplified [30]. 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). PCR settings were adjusted according to Enright et al. (2000) [30] 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.

Resistance profiles

The antimicrobial resistance profiles (AR) 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 [31]. 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) [32].

2.2.6 Statistical analysis

The 95% confidence intervals 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 [33]. 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 a $P<0.05$.

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

2.3 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 2).

The multiplex real-time PCR assay using primary and a secondary enrichment samples, 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% (CI95%: 30.6-52.6%) in animals and 51.8% (CI95%: 42.5-61.0%) in raw meat samples respectively; a significantly lower rate of 11.1% (CI95%: 4.5-24.1%) was observed in deli meat ($P \leq 0.05$). Using the primary enrichment samples and real-time PCR, a significantly higher recovery of *S. aureus* ($P \leq 0.05$) was found in animals (55.8%, CI95%: 44.8-66.9%) and raw meat (57.1%, CI95%: 47.9-66.3%) than in deli meat samples (8.9%, CI95%: 3.1-21.4%). However, no significant difference ($P > 0.05$) was found between the rate of positive *S. aureus* samples in animals (53.2%, CI95%: 42.1-64.4%), raw meat (53.6%, CI95%: 44.3-62.8%) and deli meat (42.2%, CI95%: 27.8-56.7%), when the secondary enrichment samples were tested by real-time PCR. A significantly higher recovery of *S. aureus* ($P \leq 0.05$) was obtained from deli meat when the secondary enrichment samples were assessed by real-time PCR.

The *mecA* gene was detected in two pork samples (5.4%, CI95%: 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 four pork meat and four deli meat samples. Using the primary enrichment, the real-time PCR detected the *mecA* gene in one sample isolated from a sheep, and two 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, two 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 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 *kappa* statistic for real-time PCR using the primary enrichment samples were 85.7% (*k*=0.72, CI95%: 0.62-0.82), 83.9% (*k*=0.68, CI95%: 0.59-0.76), and 97.8% (*k*=0.88, CI95%: 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 *kappa* statistic were 88.3% (*k*=0.77, CI95%: 0.67-0.86), 87.5% (*k*=0.75, CI95%: 0.67-0.83), and 68.9% (*k*=0.29, CI95%: 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.

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 4. Total agreement for real-time PCR using the primary and secondary enrichment samples ranged from 91.1% to 98.7% and from 86.7 to 98.7%, respectively. The *kappa* statistic was zero when the *mecA* gene was not detected by the conventional culture/PCR method and 0.49 (CI95%: 0.39-0.58) for raw meat. Positive agreement (sensitivity) of 100% was obtained for raw meat samples for both methods.

The real-time PCR assay 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, 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, two from pork meat) tested positive for the *nuc* gene when primary and secondary enrichments were assessed by real-time PCR.

Table 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 of ST9. A total of twenty-two *S. aureus* isolates exhibited multi-drug 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.

2.4 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 2). The inclusion of selective enrichment steps has been found to increase the rate of detection of *S. aureus* [15]. Waters *et al*. (2011) [26] 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.

The *kappa* statistic for detection of *S. aureus* using the primary enrichment in real-time PCR was 0.68-0.88 (Table 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 *kappa* statistic for detection of *S. aureus* was 0.29-0.77, 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 2), and the lower negative agreement (specificity) obtained with this method (Table 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 [23]. 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 [21,22,34,35]. 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 *kappa* statistic for both enrichments in real-time PCR was 0-0.49 (Table 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 2). Weese *et al.* (2010) [25] 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 [21,36-38]. 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* [39] suggesting that its

use in a rapid screening technique would need further validation to avoid false-positive MRSA data being generated. 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 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 genes using both methods the conventional multiplex PCR and the real-time PCR. A similar observation was reported by Weese *et al*. (2010) [25], who also failed to detect PVL positive samples in raw meat in Canada using the real-time PCR technique. The PVL genes encode the Panton-Valentine leukocidin toxin, which is a virulence factor that have been found in severe cases of CA-MRSA [7,8,9].

Decreasing the time of detection of *S. aureus* and MRSA has become an important goal in microbiological analysis of clinical samples. However, since *S. aureus* ST398, multi-drug resistant *S. aureus* (Table 5), and MRSA are present in animals and meat [12-17], decreasing the time of analysis may allow for prompt action to take place thus reducing the spread of those strains in 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 6-7 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 follow on studies. Some real-time PCR assays have been developed for the rapid detection of MRSA from clinical samples [37,40-42]. Danial *et al*. (2011) [42] 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) [40] 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) [41] 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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Conflict of interest

No conflict of interest declared.

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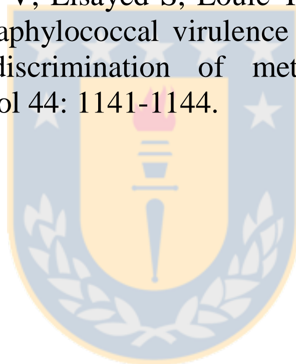

Table 1 (Desarrollo 2). Nucleotide sequence of the primers and probes used in conventional multiplex PCR, multiplex real-time PCR.

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) [43].
†Multiplex real-time PCR, according to McDonald *et al*. (2005) [35].

Table 2 (Desarrollo 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 (No. of positives)			Real-time PCR Primary enrichment (No. of positives)			Real-time PCR Secondary enrichment (No. of positives)		
		S.aureus	*mecA*	PVL	*S.aureus*	*mecA*	PVL	*S.aureus*	*mecA*	PVL
Animals										
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 (Desarrollo 2). 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.

Comparison within each sample type	No. of samples	No. positive by culture/PCR method	No. (%) of samples*			*kappa* statistic
			Positive agreement (Sensitivity)	Negative agreement (Specificity)	Total agreement	
Real-time PCR primary enrichment						
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.

Table 4 (Desarrollo 2). 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.

Comparison within each sample type	No. of samples	No. positive by culture/PCR method	No. (%) of samples*			*kappa* statistic
			Positive agreement (Sensitivity)	Negative agreement (Specificity)	Total agreement	
Real-time PCR primary 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	-	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.

Table 5 (Desarrollo 2). 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** Pork-ST9 (1)
PEN-LINC-STR	4	1	Pig-ST9 (1)
PEN-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	1	3	Pork-ST5 (1) Pork -ST5 (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 chickenChicken-ST39 (1)
LINC	1	5	Pig-ST9 (3) Sheep-ST133 (1) Deli ham-ST15
Susceptible to all tested	0	3525	Chicken-ST5 (1513) Chicken-ST6 (32) 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	

*Antimicrobial abbreviations are as following: CHL, chloramphenicol; ERY, erythromycin; LINC, lincomycin; PEN, penicillin; STR, streptomycin, TET, tetracycline, TYL, tylosin.
†ST, sequence type. ‡NT, non-typeable. **mecA* gene positive.

3. *Staphylococcus aureus* harboring *mecA* gene and genes encoding Panton-Valentine Leukocidin (PVL) exotoxin from humans

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) harbors the *mecA* gene, which encodes the low-affinity penicillin-binding protein 2a (PBP2a), and most community-associated MRSA (CA-MRSA) strains harbor genes encoding the Panton–Valentine leukocidin (PVL) exotoxin. The aim of this study was to determine the occurrence of *S. aureus* harboring *mecA* and PVL genes in hospital patients and healthy people, in North Dakota, United States. A total of 108 MRSA isolates recovered from hospital patients were examined. Nasal swabs were taken from 550 healthy subjects, and *S. aureus* was isolated and identified by culture method and biochemical testing. Multiplex PCR (mPCR) was used to confirm *S. aureus* and to detect the *mecA* and PVL genes. A total of 105 (97.2%) clinical isolates harbored the *mecA* gene and 11 (10.2%) PVL genes. The prevalence of nasal carriage of *S. aureus* in healthy people was 7.6%. None of the *S. aureus* strains obtained from healthy people were *mecA*- nor PVL-positive. Therefore, *Staphylococcus aureus* harboring *mecA* and PVL genes is present in patients affected by invasive infections, and the nasal carriage of *S. aureus* in healthy humans seems to be low, without the presence of *mecA* and PVL genes, representing a low risk of transmission among the community.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), *mecA*, PVL, prevalence, clinical isolates, healthy humans.

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3.1 Introduction

During the last 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 that are known to rapidly develop a resistance to antimicrobial agents since new antibiotics are introduced (Lowy, 2003).

A few years after the introduction of penicillin for clinical use, the first penicillin-resistant *S. aureus* was found. Methicillin-resistant *S. aureus* (MRSA) strains were identified in clinical specimens in 1961, within one to two years of the introduction of methicillin (Jevons, 1961; de Lencastre et al., 2007).

Methicillin-resistant *S. aureus* has been implicated in both community-associated (CA-MRSA) and healthcare-associated (HA-MRSA) infections worldwide. Nasal carriage of *S. aureus* and MRSA in humans in the United States was approximately 29% (78.9 million people) and 1.5% (4.1 million people) respectively, in 2003-2004 (Gorwitz et al., 2008). In 2005, there were an estimated 478,000 hospitalizations that corresponded to *S. aureus* infections, of which approximately 278,000 hospitalizations were attributed to MRSA infections (Klein et al., 2007). In addition, an invasive MRSA infection was developed by about 94,000 people, of which approximately 19,000 died. Of these infections, about 86% were HA-MRSA and 14% were 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 (Benoit et al., 2008; D'Agata et al., 2009; Nimmo et al., 2013).

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). Most CA-MRSA strains harbor genes encoding the Panton–Valentine leukocidin (PVL) exotoxin (Baba et al., 2002; Dufour et al., 2002), which has been related to severe skin infections and necrotizing pneumonia (Ebert et al., 2009).

The objective of this study was to determine the occurrence of *S. aureus* harboring *mecA* and PVL genes in hospital patients and healthy people, in North Dakota, United States.

3.2 Materials and Methods

3.2.1 Samples

A total of 108 MRSA isolates recovered from hospital patients with wound or blood stream infections (sepsis, bone, cerobrospinal fluid [CSF], synovial fluid, subdural fluid, tissue, leg ulcer and pleural fluid) were obtained from Bismarck State Hospital, Bismarck, ND, in July 2010.

In addition, during the Fall Semester 2010 and the Spring Semester 2011 a total of 550 samples were obtained from undergraduate students enrolled in the Department of Veterinary and Microbiological Sciences, North Dakota State University (ND, USA), who were considered as healthy people. Nasal swabs were taken from the subjects 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 were excluded. Presumptive *S. aureus* colonies in MSA were confirmed using Sensititre Gram Positive ID (GPID) plates (Sensititre®, TREK Diagnostic Systems Ltd., Cleveland, OH), according to the manufacturer's recommendations.

MRSA clinical isolates and confirmed *S. aureus* isolates from healthy people were stored at -80°C in brain–heart infusion broth (BD) containing 20% glycerol until use.

3.2.2 Multiplex polymerase chain reaction (mPCR)

All *S. aureus* strains were recovered from frozen stock to trypticase soy agar (TSA) plates (BD) and incubated at 37°C for 18-24 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.

Multiplex PCR assay for the detection of 16S rRNA (identification of *S. aureus*), *mecA* (detection of MRSA) and PVL-encoding genes (Table 1) included 2 µL of the DNA template (described above) added to a 50 µL final reaction mixture: 1X Go Taq® Reaction Buffer (pH 8.5), 0.025 U/µL 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), according to the protocol described by Makgotlho et al. (2009).

PCR amplicons (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 positive control (ATCC 35591) were included on each gel. Bands were visualized using an Alpha Innotech UV imager (FluorChem™).

3.2.3 Statistical analysis

The Chi-square test was used to assess the significance in prevalence of 16S rRNA, *mecA*, and PVL-encoding genes in isolates 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, 2007). On the contrary, Fisher's exact test was used with a two-sided p-values. SAS software version 9.2 (SAS Institute Inc., Cary, NC) was used to assess significance with a $P<0.05$.

3.3 Results

All clinical isolates were confirmed as *S. aureus* strains by the detection of 16S rRNA gene using the mPCR technique. Previously, these clinical isolates had been identified as MRSA strains in the hospital using microbiological procedures. Among the 108 MRSA clinical isolates, a total of 105 (97.2%) harbored the *mecA* gene and 11 (10.2%) harbored PVL genes. The mPCR method did not detect the PVL genes in MRSA strains isolated from hospital patients affected by wound infections (Table 2).

The prevalence of nasal carriage of *S. aureus* in healthy people was 7.6%. There were not significant differences ($P \geq 0.05$) between the seasons of sampling. None of these isolates harbored the *mecA* gene nor PVL genes.

3.4 Discussion

Three clinical isolates identified as MRSA in the hospital did not harbor the *mecA* gene. This may due to false positive results obtained by the microbiological method in the hospital or the presence of the novel *mecA* homolog gene (*mecA*$_{\text{LGA251}}$ renamed as *mecC*) that has been detected in MRSA isolated from humans and livestock that had been *mecA*-negative in recent studies (García-Álvarez et al., 2011; Ito et al., 2012; Laurent et al., 2012; Monecke et al., 2013; Petersen et al., 2013). The proportion of MRSA in relation to all *S. aureus* strains causing infections is still unknown, difficulting the accurate estimations of the magnitude of MRSA infections to take the right actions in public health policies (Klevens et al., 2007; Moxnes et al., 2013).

In this study, the virulence factor PVL, that encodes the PVL exotoxin in *S. aureus* related to severe skin infections and pneumonia, was detected solely in 11.1% of MRSA isolates from patients affected by blood stream infections. MRSA isolates from patients affected by wound infections did not harbor the PVL genes. Although the PVL genes were considered as a stable marker for CA-MRSA, some CA-MRSA strains have been found to be PVL-negative (Nimmo et al., 2013).

The isolation and identification of *S. aureus* strains in healthy people were carried out using a culture procedure followed by a biochemical testing (Sensititre®). The detection of *S. aureus* positive colonies obtained by the biochemical testing agreed in 100% with the results obtained by the mPCR method targetting the 16S rRNA gene. Therefore, both methods can be used for confirmation of *S. aureus* strains.

In this study, the nasal carriage of *S. aureus* in healthy people was around 7.6%, which is considerably lower than the prevalence found in other studies, that have reported a prevalence of *S. aureus* in the community around 29-32% (Mainous et al., 2006; Gorwitz et al., 2008) and among healthcare workers and patients between 29-79% (Kumar et al., 2011; Al-Talib et al., 2013). *S. aureus* strains isolated from healthy people did not harbor the *mecA* and PVL genes, representing a low risk of transmission in the community. Other studies have reported a nasal carriage of MRSA approximately of 0.8-1.5% in the community (Mainous et al., 2006; Gorwitz et al., 2008), 0.5-44% in patients (Tiemersma et al., 2004), 20% in healthcare workers (Kumar et al., 2011) and 30% in people living and working in farms with MRSA-positive pigs or dust (Van den Broek et al., 2009). Studies that reported a high prevalence of *S. aureus* and MRSA in the community have considered a higher sample size of subjects, with different demographic characteristics (age, race/ethnicity, sex, place of birth) and with samplings in different years. In addition, the high prevalence of *S. aureus* and MRSA among healthcare workers and patients is due to the exposure to hospital environment with major risks in transmitting and spreading nosocomial infections.

3.5 Conclusion

Staphylococcus aureus harboring *mecA* and PVL genes is present in patients affected by invasive infections. The nasal carriage of *S. aureus* in healthy humans seems to be low, and the *mecA* and PVL genes could not be present. Therefore, MRSA might not represent a severe risk for transmission among the community.

Further molecular typing techniques are needed to improve the molecular characterization of the *S. aureus* isolates obtained from humans.

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Table 1 (Desarrollo 3). Nucleotide sequence of the primers used in the multiplex PCR for detection of the 16S rRNA gene, the *mecA*gene and the PVL genes (McClure et al., 2006).

Primer	Oligonucleotide sequence	Amplicon Size (bp)
Staphy 756 F Staph 750 R	5'-AAC TCT GTT ATT AGG GAA GAA CA-3' 5'-CCA CCT TCC TCC GGT TTG TCA CC-3'	756
mecA-1 F *mecA*-2 R	5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3' 5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3'	310
luk-PV-1 F *luk*-PV-2 R	5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3' 5'-GCA TCA AGT GTA TTG GAT AGC AAA AGC-3'	433

Table 2 (Desarrollo 3). Identification of 16S rRNA, *mecA* and Panton-Valentine Leukocidin (PVL) genes in *Staphylococcus aureus* isolates from healthy people and methicillin-resistance *Staphylococcus aureus* (MRSA) isolates from hospital patients.

Source	No. of samples	No. of samples positive for *S. aureus* (%)	No. of samples positive for MRSA (%)	No. of isolates with the specific gene (%)		
				16S rRNA	*mecA*	PVL
Hospital patients						
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)
Healthy people						
Fall 2010	231	17 (7.4)		17 (7.4)	0 (0.0)	0 (0.0)
Spring 2011	219	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)

III. DISCUSIÓN GENERAL

En este estudio se encontró una alta prevalencia de *S. aureus* en animales y carne cruda (35% y 48%, respectivamente), observándos una mayor prevalencia en corderos y cerdos y en las carnes de pollo y cerdo. Sin embargo, en productos cárnicos la prevalencia de *S. aureus* fue menor (13%). En personas sanas, se obtuvo una baja proporción de portadores de *S. aureus* (8%), cuando se compara con los resultados de otros estudios (Mainous et al., 2006; Gorwitz et al., 2008). Adicionalmente, se detectó la presencia del gen *mecA* sólo en carne de cerdo, con una baja prevalencia (3%) similar a lo reportado en otras investigaciones (de Boer et al., 2009; Pu et al., 2009). Finalmente, se determinó en animales (cerdos y corderos) y carne de cerdo la presencia de *S. aureus* MR y *S. aureus* ST398.

La presencia de SARM, *S. aureus* MR y *S. aureus* ST398 en animales de abasto, en carne y productos cárnicos, sugiere que las personas podrían estar expuestas a adquirir este tipo de agentes de infección a través de la cadena productiva de la carne, existiendo un riesgo mayor cuando se trata de productos cárnicos que no requieren cocción previa al consumo.

En este estudio no se detectaron los genes PVL en ninguna de las cepas de *S. aureus* aisladas de animales, carne y personas sanas. Sólo algunas cepas de SARM obtenidas de pacientes enfermos hospitalizados (10%) resultaron ser positivas para estos genes de virulencia.

Los resultados de prevalencia de *S. aureus* obtenidos en animales y carne mediante el método de cultivo coincidieron con los resultados obtenidos a través de la técnica de PCR (identificación del gen ARNr 16S). Por esta razón, resulta conveniente incluir una etapa de enriquecimiento selectivo previo a la etapa de cultivo selectivo, seguido de la confirmación de las cepas presuntivas de S. *aureus* a través de pruebas bioquímicas.

En este estudio la detección de SARM se determinó a través de la técnica de PCR (identificación del gen *mecA*) y test de susceptibilidad antimicrobiana (resistencia a antibióticos β-lactámicos). Existen otros métodos que se han utilizado para confirmar la presencia de SARM. Estos métodos incluyen tests de susceptibilidad antimicrobiana a la oxacilina o cefoxitina (Danial et al., 2011; Kumar et al., 2011; Kim et al., 2013; Nimmo et al.,

2013) o detección de la proteína PBP2a a través de tests de aglutinación (Anderson and Weese, 2007; Weese et al., 2010; Danial et al., 2011).

El método utilizado para aislar *S. aureus* y SARM puede influir en los resultados de prevalencia. Algunos autores han utilizado sólo una etapa de cultivo en placa (Weese et al., 2006; Aydin et al., 2011), y otros han incluido etapas de enriquecimiento selectivo previo a la etapa de cultivo (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) utilizando las etapas de enriquecimiento primario y secundario, como se describe en el presente trabajo, obtuvieron una mayor detección de SARM en muestras de carne. Tanto las cepas de *S. aureus* y SARM pueden ser aisladas utilizando medios selectivos que contienen NaCl. Sin embargo, algunas cepas de SARM no crecen cuando la concentración excede de 2,5% (Jones et al., 1997). Además, algunos medios suplementados con antibióticos podrían causar un crecimiento exacerbado de cepas de *S. aureus* susceptibles a la meticilina (Böcher et al., 2008) o fallar en el aislamiento de SARM. Por esta razón, se recomienda incluir siempre un medio de cultivo libre de antibióticos, aunque el objetivo sea la identificación de SARM (Pu et al., 2009).

La rápida detección de *S. aureus* y SARM en animales y carne permitiría disminuir el riesgo de exposición a contaminación en la cadena productiva de la carne. En este estudio para disminuir los tiempos de detección, se desarrolló un ensayo de PCR múltiple en tiempo real con el fin de detectar los genes *nuc* (identificar *S. aureus*), *mecA* (asociado a SARM) y PVL (factor de virulencia), en muestras extraídas de animales y carne. Se encontró una concordancia total alta entre el método de cultivo convencional y la técnica de PCR en tiempo real, con una mayor detección al utilizar el medio de enriquecimiento secundario. Lo anterior se debería a la recuperación de células dañadas al utilizar el enriquecimiento secundario y a que niveles bajos de *S. aureus* no serían detectados al utilizar sólo el enriquecimiento primario en PCR en tiempo real. Sin embargo, la técnica de cultivo convencional sigue siendo considerada la metodología estándar para la identificación de *S. aureus* (Huletsky et al., 2004; Paule et al., 2005; Danial et al., 2011) y PCR convencional (detección del gen *mecA*) para la identificación de SARM (Maes et al., 2002; Makgotlho et al., 2009).

Para la técnica de PCR en tiempo real, se utilizó ADN extraído de los caldos de enriquecimiento de las muestras obtenidas de animales y carne, por lo cual la concentración y procedencia del material genético fue variable. Se detectó la presencia del gen *mecA* en muestras que resultaron ser negativas para *S. aureus*, pero positivas para *Staphylococcus* spp. a través del método de cultivo y test bioquímicos. Estos resultados son considerados falsos positivos, ya que se ha detectado el gen *mecA* tanto en *S. aureus* como en cepas staphylococci coagulasa-negativa (Ryffel et al., 1990; Hagen et al., 2005; Higashide et al., 2006; Thomas et al., 2007; Black et al., 2011). Desafortunadamente, en este estudio los resultados no pudieron ser validados a través del método de cultivo, ya que se utilizó extractos de ADN, habiéndose inactivado las células previamente.

El método de PCR en tiempo real utilizado en este estudio debió evaluarse en términos de sensibilidad de detección, determinando la concentración mínima de ADN para obtener amplificación. Además de utilizar las muestras control externo, se recomienda incorporar un control interno de amplificación que podría indicar resultados falsos-negativos, por la presencia de inhibidores en la reacción, mal funcionamiento del termociclador, actividad baja de la polimerasa o incorrecta solución de PCR (Hoorfar et al., 2004).

La similitud genética encontrada entre las cepas de *S. aureus* aisladas de cerdos y carne de cerdo (ST9), sugiere la posible contaminación de la carne durante la etapa de la faena. De las cepas positivas para el gen *mecA*, dos resultaron ser ST5, y tres ST398, todas encontradas en carne de cerdo. Además, se encontró una alta prevalencia de *S. aureus* ST398 (no-SARM), lo cual indica un riesgo potencial para las personas de adquirir esta infección a través de la cadena productiva de la carne.

Para la tipificación molecular de *S. aureus* a través de PFGE se sigue el protocolo de PulseNet, que utiliza la enzima de restricción *Sma*I. Sin embargo, esta enzima no puede digerir el ADN de las cepas ST398, debido a que presentan una enzima que metila la secuencia de reconocimiento de *Sma*I (Bens et al., 2006). Por lo tanto, no es posible obtener un patrón de restricción con PFGE para ser comparado con otras cepas. Por esta razón, en este estudio se utilizó una segunda enzima de restricción, *Xma*I (un isoesquizómero de *Sma*I), con la cual se obtuvo patrones de restricción con bandas muy débiles de las cepas ST398. Para poder realizar

un análisis más preciso de la tipificación molecular de *S. aureus* ST398 con PFGE, se debe buscar enzimas de restricción que actúen sobre otras secuencias de reconocimiento, diferentes a las enzimas *Sma*I y *Xma*I, y así establecer un protocolo de PFGE para las cepas que no presenten un patrón de restricción definido.

Para tener mayor precisión en la tipificación molecular de las cepas, se recomienda la utilización de al menos dos técnicas moleculares (Tenover et al., 1994). En este estudio se utilizó la técnica PFGE y MLST en la tipificación de las cepas de *S. aureus*. Generalmente, PFGE y MLST clasifican las cepas en clusters similares (Catry et al., 2010). Otro método de tipificación utilizado para *S. aureus* es la tipificación de *spa*, la cual tiene como desventaja de que líneas clonales no relacionadas pueden tener *spa* tipos similares (Van den Broek IV et al., 2009; Golding et al., 2008). Por esta razón, se podrían obtener discrepancias con respecto a los resultados a través de MLST y PFGE (Golding et al., 2008). Sin embargo, el método de tipificación molecular con mayor poder de discriminación es PFGE (McDougal et al., 2003).

Todas las cepas SARM detectadas en este estudio resultaron ser resistentes a la penicilina a través del test de susceptibilidad antimicrobiana, obteniéndose una CMI mayor que en las otras cepas de *S. aureus*. Además, se obtuvo una alta prevalencia de *S. aureus* MR [resistente al menos a tres agentes antimicrobianos (Aydin et al., 2011)] en animales y en carne cruda, principalmente a los antibióticos penicilina, tetraciclina, lincomicina y eritromicina. La multirresistencia se presentó principalmente en cepas ST398 y ST9, las cuales se asocian a animales, específicamente a cerdos (Lewis et al., 2008; van Belkum et al., 2008; Guardabassi et al., 2009; Krziwanek et al., 2009).

Los agentes antimicrobianos son drogas efectivas contra los agentes infecciones. Sin embargo, su uso imprudente contribuye a la resistencia bacteriana, ya sea en hospitales, en la comunidad o en producción animal. El uso extensivo de agentes antimicrobianos ejerce una presión selectiva sobre las cepas resistentes, eliminando los competidores susceptibles (Swartz, 1997; Marinelli y Tomasz, 2010). Los glicopéptidos, como la vancomicina, son frecuentemente utilizados para el tratamiento de infecciones causadas por SARM, sin embargo, en los últimos años ha aumentado la incidencia de *S. aureus* con resistencia y resistencia intermedia a este antibiótico (Tiwari y Sen, 2006). En este estudio todas las cepas de *S. aureus*

aisladas de animales y carne fueron susceptibles a vancomicina, así como también a daptomicina, linezolida y nitrofurantoina, coincidiendo con los resultados obtenidos por Pu et al. (2011) en muestras de carne. Los estudios anteriores y los resultados de este estudio sugieren que las infecciones por *S. aureus* y SARM originarios de animales portadores o de carne contaminada podrían ser tratadas con daptomicina, linezolida o nitrofurantoina por la susceptibilidad que presentan las cepas.

En producción animal, los antibióticos se utilizan como profilácticos, en el tratamiento de enfermedades y como promotores del crecimiento (DuPont y Steele, 1987; Franco et al., 1990). Esto puede generar patógenos resistentes en animales, existiendo el riesgo potencial de que los genes de resistencia sean incorporados en bacterias presentes en humanos. En este estudio, la identificación de *S. aureus* MR en animales y en carne plantea el riesgo de transmisión a las personas a través de la cadena de la carne y explicaría la disminución de la eficacia de este tipo de medicamentos utilizados en salud humana (Smith et al., 2002).

Se recomienda que los hospitales disminuyan la utilización de un antibiótico antes de que las cepas resistentes se propaguen. Sin embargo, puede ocurrir que el gen de resistencia haya sido transpasado a plásmidos que portan resistencia a agentes antimicrobianos utilizados como alternativas de la droga original (Swartz, 1997). Se debe aumentar los esfuerzos para reducir el uso de antibióticos, asegurar el control de las infecciones y los mecanismos de vigilancia, y seleccionar las dosis y combinaciones utilizadas, para evitar la emergencia de resistencia (Marinelli y Tomasz, 2010).

En la actualidad se están desarrollando nuevos agentes antimicrobianos que sean efectivos contra los patógenos resistentes. Las investigaciones que se desarrolllen en este ámbito debieran considerar la evaluación de la susceptibilidad de *S. aureus* a estos nuevos antimicrobianos y combinaciones de ellos y, además, investigar aún más acerca de los diferentes mecanismos de resistencia que pueden presentarse en los microorganismos. De esta forma, se podrá contar con la información necesaria para poder controlar *S. aureus* resistente y disminuir la exposición de las personas en las diferentes etapas de la cadena productiva de la carne.

En Estados Unidos existe un sistema de vigilancia llamado National Antimicrobial Resistance Monitoring System (NARMS), creado en 1996 en colaboración con FDA (Food and Drug Administration), CDC, USDA (U.S. Department of Agriculture) y los departamentos de salud estatales y locales. NARMS realiza un monitoreo de la susceptibilidad antimicrobiana de bacterias entéricas aisladas en humanos, carne y animales de producción. Los patógenos que se incluyen en NARMS son *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., *Shigella* spp. y *Enterococcus* spp. (FDA, 2012). Sin embargo, la evidencia que existe de la presencia de SARM y *S. aureus* MR en la cadena productiva de la carne, sugiere que se debiera ampliar el monitoreo a otros patógenos resistentes, no sólo las bacterias entéricas que se incluyen actualmente. De esta forma, al incluir *S. aureus* en este sistema nacional de monitoreo se podría conocer año a año la prevalencia de las cepas resistentes, la susceptibilidad antimicrobiana que presentan, evaluar la susceptibilidad a nuevos agentes antimicrobianos y establecer estrategias de control y mitigación para disminuir la exposición de las personas.

En este estudio se analizaron cepas SARM obtenidas de pacientes enfermos, de las cuales tres fueron negativas para el gen *mecA*. Esto podría deberse a la obtención de resultados falsos positivos en el hospital o a la presencia de un nuevo homólogo del gen *mecA*, recientemente identificado como *mecC*, el cual ha sido detectado en SARM negativo para el gen *mecA*, aislado de humanos y animales (García-Álvarez et al., 2011; Ito et al., 2012; Laurent et al., 2012; Monecke et al., 2013; Petersen et al., 2013). Además, se extrajeron muestras de personas sanas para determinar la proporción de portadores de SARM sanos, resultando ser negativo para todos los individuos. Para obtener una mayor precisión en la identificación y caracterización de SARM en estas muestras, se recomienda la detección de ambos genes (*mecA* y *mecC*), además de la determinación de la susceptibilidad antimicrobiana, y tipificación molecular a través de PFGE y MLST.

IV. CONCLUSIONES GENERALES

De los resultados obtenidos en este estudio se puede concluir que:

Existe una alta prevalencia de *S. aureus* en animales productores de carne y carne cruda y una baja proporción de personas portadoras sanas. Las cepas de *S. aureus* aisladas de animales, carne y personas sanas en este estudio no son portadoras de los genes PVL. Cepas de *S. aureus* extraídas de pacientes enfermos que padecen infecciones presentan los genes *mecA*, asociado a la resistencia y PVL, asociado a la virulencia. A pesar de que la prevalencia de SARM en carne cruda es baja, la alta prevalencia de *S. aureus* MR, y cepas ST398 en la cadena productiva de la carne, sugiere que existe un riesgo de transmisión a las personas. Además, la similitud genética entre las cepas de *S. aureus* encontradas en animales y carne, sugiere la contaminación de la carne durante la faena.

Se recomienda incorporar la detección de SARM y *S. aureus* MR y la determinación de la susceptibilidad antimicrobiana de las cepas en los sistemas de vigilancia que existen a nivel federal, como es NARMS (National Antimicrobial Resistence Monitoring System), considerando el monitoreo de la cadena de la carne, de pacientes en los hospitales y de personas sanas en la comunidad.

La utilización de medios de enriquecimiento selectivos previo a la etapa de cultivo permite aislar *S. aureus* y SARM, obteniéndose 100% de concordancia con la técnica de PCR convencional. La utilización de un medio de enriquecimiento secundario junto con PCR en tiempo real mejora la detección de *S. aureus* en muestras de animales y carne. La técnica de PCR en tiempo real permite una detección rápida de *S. aureus* y el gen *mecA*, siendo recomendable confirmar la presencia de SARM a través de la metodología estándar.

Para establecer medidas de control de la propagación de las cepas resistentes de *S. aureus* en la cadena productiva de la carne es necesario determinar la tipificación molecular y la susceptibilidad antimicrobiana.

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