Epidemiological investigation into the possible exchange of SCC*mec* between staphylococci in different ecosystems

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"If the problem can be solved why worry? If the problem cannot be solved worrying will do you no good."

Śāntideva

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List of abbreviations

ABC: ATP binding cassette

ACME: arginine catabolic mobile element

BURP: based upon repeat pattern

BURST: based upon related sequence types

CA-MRSA: community associated MRSA

CC: clonal complex

ccr: cassette chromosome recombinase

CHL: chloramphenicol

CI : confidence interval

CIP : ciprofloxacin

CLI : clindamycin

CoNS: coagulase negative staphylococci

CP: capsular polysaccharide

CSB: Columbia Sheep Blood

DBEM: double broth enrichment method

DNA: deoxyribonucleic acid

ECOFF: epidemiological cut-offs

EDTA: Ethylenediaminetetraacetic acid

EF-G: elongation factor G

EFSA: European Food Safety Authority

ERY: erythromycin

EUCAST: European Committee on Antimicrobial Susceptibility Testing

ET: exfoliatins

FASFC: Federal agency for the safety of the food chain

FOX: cefoxitin

FUS : fusidic acid

GEN : gentamicin

HA-MRSA: hospital acquired MRSA

IEC: immune evasion cluster

J-region: joining region

KAN: kanamycin

kb: kilobase

LA-MRSA: livestock associated MRSA

LR+: likelihood ratio positive

LR-: likelihood ratio negative

LZD: linezolid

MALDI-TOF: matrix-assisted laser desorption ionization time-of-flight

MGE: Mobile genetic element

MH: Mueller-Hinton

MIC: minimal inhibitory concentration

MLS_B: macrolides, lincosamides and streptogramin B

MLST: multi locus sequence typing

MRCoNS: methicillin resistant coagulase negative staphylococci

MRSA: methicillin-resistant *Staphylococcus aureus*

MRSS: methicillin-resistant *Staphylococcus sciuri*

MS_B: macrolides and streptogramin B

MSCRAMM: microbial surface components recognizing adhesive matrix molecules

MUP: mupirocin

M-PCR: multiplex Polymerase chain reaction

ND: not determined

NPV: negative predictive value

NS: not specified

NT: non-typeable

ORF: open reading frame

PBP: penicillin-binding protein

PCR: polymerase chain reaction

PEN : penicillin

PFGE: pulsed field gel electrophoresis

PPV: positive predictive value

PVL: Panton-Valentine leukotoxin

RIF : rifampicin

rRNA: ribosomal ribonucleic acid

SBEM: single broth enrichment method

SCC*mec*: staphylococcal cassette chromosome *mec*

SE: staphylococcal enterotoxin

SMX: sulfamethoxazole

ST: sequence type

STR : streptomycin

SYN : quinupristin/dalfopristin

TET : tetracyclin

TIA : tiamulin

TMP : trimethoprim

Tn: transposon

tRNA: transfer ribonucleic acid

TSB: tryptic Soy Broth

TSST: toxic shock syndrome toxin

UPGMA: unweighted pair group method with arithmetic mean

USA: United States of America

VAN : vancomycin

Preface

Antimicrobial resistance in staphyococci became a concern in medicine since its discovery in hospitals during the Wold War II, with the first apparition of penicillin resistant *Staphylococcus aureus*. Attention toward this resistant bacterium increased with appearance of resistance against new antimicrobials such as methicillin in the 1960s. The burden of methicillin resistant *S. aureus* (MRSA) kept increasing with its discovery in the community and later in livestock animals. MRSA were then broadly studied in many countries and its great adaptive ability was highlighted by the development of resistance against all classes of antimicrobials, including β-lactams, used in human or veterinary medicine.

In parallel to this, interest in other staphylococci starts growing and bacteria that have long been considered as harmless commensals were shown to be also implicated in human and animal cases of infections. However, little is still known on the diversity of these staphylococci of the coagulase negative group. Among these, *Staphylococcus sciuri*, considered as ancestral bacterium of this genus, and its closely related species, were shown to carry the putative evolutionary ancestor of the *mecA* gene encoding β-lactam resistance. Hypotheses supporting a possible transmission of resistance genes from these species to other staphylococci such as *S. aureus* were then supported though not confirmed yet.

In the framework of this research, the focus was on MRSA and methicillin resistant *Staphylococcus sciuri* (MRSS) in livestock in order to determine their prevalence in different animals and to give insight in the possible role of *S. sciuri* as a reservoir for resistance and virulence genes for *S. aureus*. To reach these aims, after an introduction on the current knowledge on MRSA and the *S. sciuri* species group (**chapter 1-3**), results on the prevalence and molecular characterisation of MRSA in poultry (**chapter 4**) and in bovines (**chapter 5**) are presented. In these chapters, MRSA prevalence will be compared in the different rearing

practices and age groups. MRSA isolates from bovines were also investigated for their antimicrobial resistance and virulence genes. The two following chapters focus on MRSS in healthy chickens (**chapter 6**) and in different farm animals (**chapter 7**). These two chapters aimed at determining the prevalence and genetic diversity of MRSS in different animal populations. Antimicrobial resistance and virulence genes were also investigated in order to have an idea of the genetic pool available in this species. In the last chapter, all results of this research are grouped and discussed.

Part I – Review of the literature

This review of the literature deals with antimicrobial resistance and epidemiology of staphylococci and is divided in three main chapters. **Chapter 1** describes general characteristics of staphylococci and briefly introduces the history of antimicrobial resistance. This chapter also presents different typing methods allowing the determination of the staphylococci population structure. **Chapter 2** focuses on MRSA in livestock animals, and discusses general characteristics of livestock associated (LA)-MRSA as well as its epidemiology in healthy and diseased animals and antimicrobial resistance and virulence genes encountered. Finally, **chapter 3** is dedicated to the *Staphylococcus sciuri* species group, its epidemiology, population structure as well as the diversity of virulence and antimicrobial resistance genes encountered in this group.

Partly adapted from:

Nemeghaire, S., Argudín, M.A., Feβler, A., Hauschild ,T., Schwarz, S., Butaye, P. The ecological importance of the *Staphylococcus sciuri* species group as a reservoir for resistance and virulence genes. Vet. Mic. doi: 10.1016/j.vetmic.2014.02.005

Chapter 1 – Review of Staphylococci

1.1. General characteristics of staphylococci

The genus *Staphylococcus* was first described by Koch and Pasteur in 1880. This genus currently comprises over 50 species and subspecies (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1279) and new species are being described continuously. Staphylococci are Gram-positive non-motile bacteria whose cell wall contains peptidoglycan and teichoic acid. They are facultative anaerobic bacteria that are usually catalase-positive and oxidase-negative (De Vos *et al*., 2009). Two major groups have been identified in the genus *Staphylococcus*; the coagulase- and DNase-positive group (including *Staphylococcus aureus*) and the coagulase negative staphylococci (CoNS) group that includes, among others, *Staphylococcus sciuri* and related species.

Among coagulase positive staphylococci*, S. aureus* is an opportunistic bacterium commonly found on mucous membranes of humans and warm blooded animals (William, 1963; Devriese *et al*., 1976). Some *S. aureus* strains easily acquire antimicrobial resistances (Livermore, 2000). Known as a common pathogen in human medicine, *S.aureus* was also found to be responsible for various infections in domestic (Vanderhaeghen *et al*., 2010a) and wild animals (Monecke *et al*., 2013a).

CoNS comprise, among others, the *S. sciuri* species group which includes five species: *S. sciuri* (with three subspecies), *Staphylococcus lentus*, *Staphylococcus vitulinus*, *Staphylococcus fleurettii* and *Staphylococcus stepanovicii* (De Vos *et al*., 2009, Hauschild *et al*., 2010). Members of these species are commonly found in a broad range of habitats including animals, humans and the environment (Kloos *et al.* 1976a; Adegoke, 1986, Pioch *et al*., 1988, Shittu *et al.,* 2004). However, those species have also been isolated from infections, both in veterinary and human medicine.

1.2. Brief history of antimicrobial resistance in staphylococci

Penicillin was first tested on soldiers suffering from staphylococcal infections during the World War II (Abraham *et al*., 1941). Few years later, resistance emerged and spread fast. The number of penicillin resistant *S. aureus* in hospitals increased quickly from 6% in 1946 to over 50% in 1948 (Livermore *et al*., 2000). Since this proportion was still increasing within the following years, other natural antimicrobials were developed (figure 1). These antimicrobials included chloramphenicol, macrolides, aminoglycosides and tetracyclines and were at first active against *S. aureus*. However, resistance against these antimicrobials emerged also quickly and multi-resistant *S. aureus* became a major problem in hospitals in the 1950s (Livermore *et al*., 2000). In the beginning of the1960s, cephalosporins and synthetic penicillinase stable β-lactams such as methicillin, nafcillin and oxacillin were introduced for their stability to staphylococcal penicillinase.

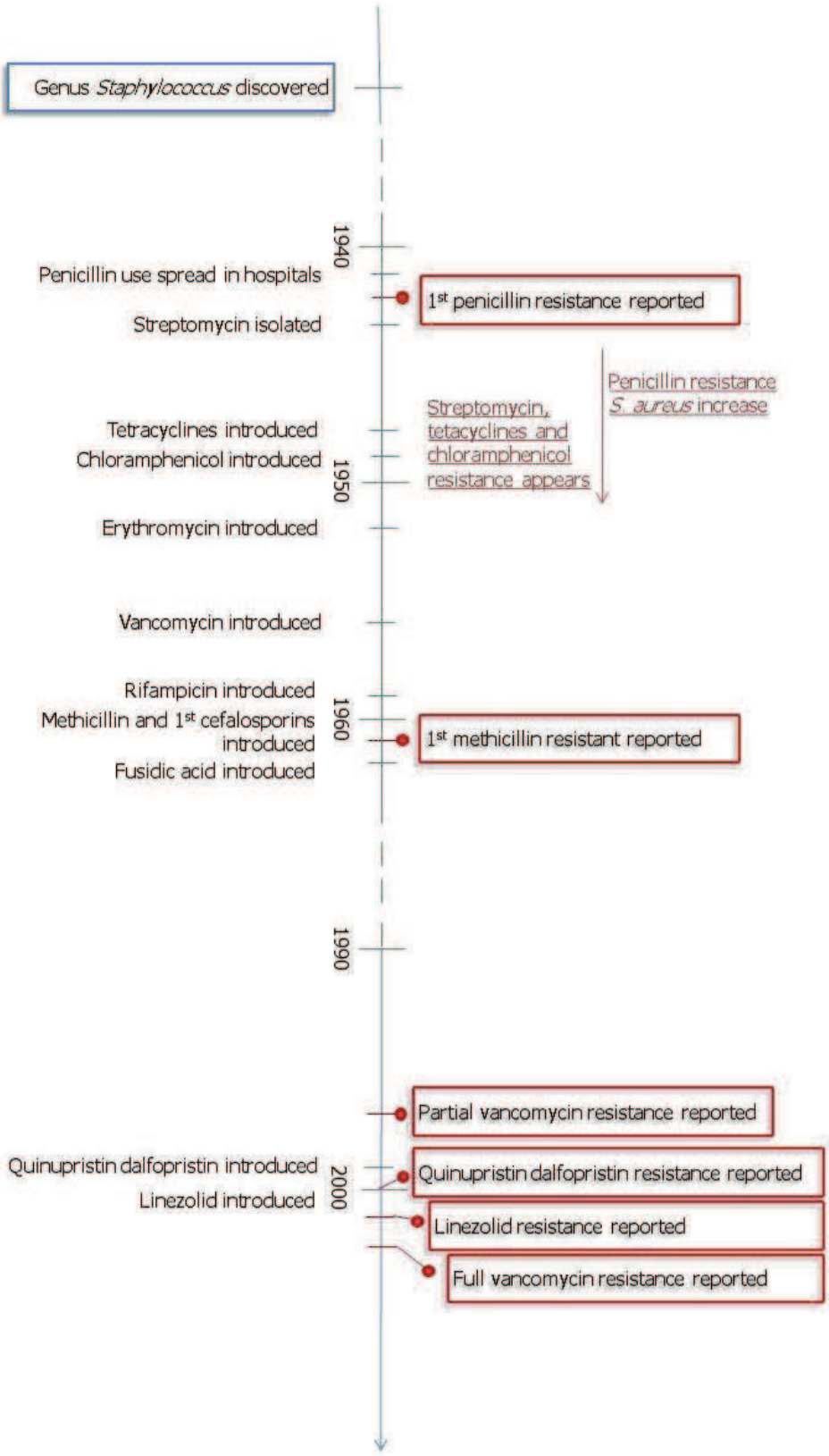

Figure 1. Time line of the discovery of main antimicrobial agents used in treatment against staphylococci (Livermore *et al*., 2000).

Resistance to the newly discovered methicillin appeared within the year of its introduction (1961). The appearance of methicillin resistant *S. aureus* (MRSA) in the early 1960s spread during the 1970s and reached 10% of *S. aureus* at a major general hospital in Birmingham and 15% of *S. aureus* from infective sources in Denmark (Livermore *et al*., 2000). The introduction of gentamicin led to a decrease of MRSA prevalence during the 1980s though gentamicin resistant *S. aureus* and MRSA began to emerge during the same period (Rouch *et al*., 1987). In the 90's, antimicrobials of the fluoroquinolone family were introduced. Shortly thereafter, MRSA isolates appeared resistant to ciprofloxacin which was extensively used. Fortunately and in contrast to other antimicrobials, this resistance seems not to spread easily and until now, the number of fluoroquinolone resistant MRSA remains low. To date, glycopeptides remain active against MRSA. Rifampicin and fusidic acid are also considered as possible alternatives though fusidic acid resistance has been widely encountered in coagulase negative staphylococci of the *S. sciuri* species group.

1.3. Molecular techniques to study the population structure of staphylococci

The population structure of staphylococci can be determined by different molecular techniques. Among these, multi locus sequence typing (MLST) and typing of the staphylococcal protein A encoding gene (*spa*) have been developed in order to type *S. aureus*. Meanwhile, pulsed field gel electrophoresis (PFGE) is more a generic method applicable to staphylococci. Additionally to this, the typing of the staphylococcal cassette chromosome *mec* (SCC*mec*) is used as a subtyping method for methicillin resistant isolates.

1.3.1. Multi locus sequence typing

MLST is a highly discriminative method for the characterization of bacterial isolates. In MLST of *S. aureus*, internal fragments of seven housekeeping genes are amplified and

sequenced (Maiden *et al.*, 1998; Enright *et al.*, 2000). The sequences are assigned as distinct alleles. Each isolate is then defined by the alleles of the seven housekeeping loci. Using the *S. aureus* MLST database (http://saureus.mlst.net/sql/sthtml.asp), a sequence type (ST) is assigned to each isolate (Enright *et al*., 2000). Strains that differ in only one or two loci are called single locus variants and double locus variants, respectively. Using "based upon related sequence types" (BURST) analysis, these sequence types and locus variants are grouped into clonal complexes (CC).

1.3.2. Pulsed field gel electrophoresis

PFGE is an electrophoresis method, in which the voltage is periodically switched among three directions. In epidemiological studies of MRSA, PFGE is one of the most widespread molecular typing methods used in DNA fingerprinting. The "gold standard" for PFGE typing of whole genome of *S. aureus* is the macroresticton using the enzyme SmaI (Tenover *et al.*, 1995; Mulvey *et al.*, 2001). However, modifications/methylations of the SmaI restriction site is frequent in the most common ST in MRSA of animal origin (ST398) and prevent the SmaI enzyme from fragmenting the DNA. This one can be replaced by the isoschizomer enzyme Cfr9I (Argudín *et al*., 2010).

1.3.3. spa type

spa typing consists in the amplification and sequencing of the polymorphic X-region of the staphylococcal protein A (*spa*). This gene contains a variable number of different repeats of mostly 24 bp (Frénay *et al.*, 1994). The sequence of each repeat and the total number of repeats determine a profile called the *spa* type (Harmsen *et al.*, 2003).

1.3.4. SCCmec

The *mecA* gene responsible for β-lactam resistance is located on a mobile genetic element (MGE) named SCC*mec*. This SCC*mec* is composed of two essential gene complexes. The *mec* complex contains the *mecA* gene and its direct regulatory genes, *mecI* and *mecR1*, and associated insertion sequences, IS431. The second complex is a cassette chromosome recombinase (*ccr*) responsible for the insertion and excision of the cassette. Those two complexes can be distinguished according to their structural composition (Hiramatsu, 1995). To date, five classes of the *mecA* gene complex (A to E) and eight *ccr* gene complexes (*ccr*A1B1, *ccr*A2B2, *ccr*A3B3, *ccr*A4B4, *ccr*C1, *ccr*A5B3, *ccr*A1B6 and *ccr*A1B3) have been defined (Katamaya *et al*., 2001; Ito *et al*., 2004). Following these combination, eleven SCC*mec* types (table 2) have been reported (http://www.sccmec.org).

Table 2. SCC*mec* types identified to date in staphylococci.

SCC*mec* type	*ccr* genes complexes	*mec* complex
I	1 (A1/B1)	B
II	2 (A2/B2)	A
III	3 (A3/B3)	A
IV	2 (A2/B2)	B
V	5 (C1)	C2
VI	4 (A4/B4)	B
VII	5 (C1)	C1
VIII	4 (A4/B4)	A
IX	1 (A1/B1)	C2
X	7 (A1/B6)	C1
XI	8 (A1/B3)	E

Additionally to these two complexes, the SCC*mec* cassette contains three non-essential joining (J) regions (Figure 2) that may contain other resistance genes.

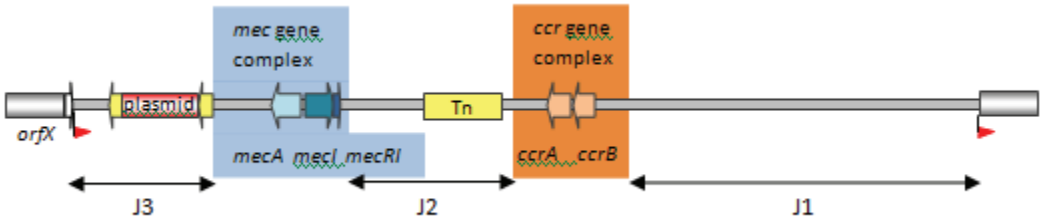

Figure 2. Basic schematic structure of the SCC*mec* element with a type A *mec* gene complex in blue and a *ccr* gene complex that contains a *ccrA* and a *ccrB* gene in orange and three joining-regions (J1-J3). The red arrowheads indicate the integration site for staphylococcal cassette chromosome (SCC) in an open reading frame (ORF) called orfX. (Vanderhaeghen, 2012a).

Identification of the complexes is investigated using simplex (Ito *et al.*, 1999; Okuma *et al.*, 2002) or multiplex (Oliveira and de Lancastre, 2002; Zhang *et al.*, 2005; Kondo *et al.*, 2007; Milheiriço *et al.*, 2007) PCR techniques. Furthermore, since the J regions show great variations (Hiramatsu *et al.*, 1992), subtyping can be performed by PCR mapping of these regions (Milheiriço *et al.*, 2007). However, all these methods mentioned referred to the typing of MRSA isolates of human origin. It has been shown that these methods may fail in determining the SCC*mec* element from MRSA of animal origin (Nemati *et al.*, 2008) or from CoNS (Vanderhaeghen *et al.*, 2012b). These SCC*mec* types are then considered non-typeable (NT). Since both MRSA and several CoNS may carry very similar SCC*mec* elements, horizontal transfer of SCC*mec* is supposed (Bloemendaal *et al.*, 2010; Smyth *et al.*, 2011; Vanderhaeghen *et al.*, 2012b; Vanderhaeghen *et al.*, 2013). However, the exact mechanisms of this potential horizontal transfer are still unknown and have never been shown in vitro (Shore *et al.,* 2005).

Chapter 2 - Methicillin resistant *Staphylococcus aureus* in livestock

2.1. General characteristics of methicillin resistant *Staphylococcus aureus* in livestock animals

In farm animals, MRSA was isolated first in 1972, from cows with mastitis in Belgium (Devriese *et al.*, 1972). Later, few other cases of MRSA in animals were reported though these isolates appeared to be of human origin and were mostly related to pet animals (van Duijkeren *et al.*, 2004). MRSA in domestic animals became a concern in 2005 when, in The Netherlands, a same MRSA strain was isolated from a child of a pig farmer and pigs on that farm (Voss *et al.*, 2005). This specific type was later found to spread not only between pigs but also to other animal such as cows (Vanderhaeghen *et al*., 2010b; Spohr *et al*., 2011), horses (Hermans *et al.*, 2008; Cuny *et al.*, 2010), dogs (Witte *et al*., 2007) and poultry (Nemati *et al*., 2008; Argudín *et al*., 2013). Subsequently, other lineages were recovered from animals. Indeed, while MRSA ST398 together with ST97, are mainly recovered from Europe and The United States (Anon. 2009; Smyth *et al*., 2009; Battisti *et al*., 2010; Gómez-Sanz *et al*., 2010; Meemken *et al*., 2010), ST9 is mainly recovered from Asian countries (Cui *et al*., 2009; Guardabassi *et al*., 2009; Neela *et al*., 2009; Wagenaar *et al*., 2009). These MRSA lineages that are commonly recovered from samples of animal origin are commonly called livestock associated (LA)-MRSA. Now, MRSA ST398 has spread in many countries all over the world (van Duijkeren *et al*., 2007; Schwarz *et al*., 2008; Meemken *et al.*, 2010; Van der Wolf *et al*., 2012; Crombé *et al*., 2012). Seen its possible transfer to human, LA-MRSA might represent a significant risk for human carriage of MRSA (Voss *et al.*, 2005).

2.2. Epidemiology

2.2.1. In healthy animals

MRSA has been recovered from various animal species in different countries. In 2009, the European Food Safety Authority (EFSA) published a report on the prevalence of MRSA in pig holdings in Europe (Anon., 2009). During this survey, it was concluded that monitoring of MRSA in livestock animal species was recommended. Since this baseline survey, numerous studies aiming to assess the prevalence and diversity of MRSA have been carried out in various livestock animal populations in a number of member states. The frequency of MRSA carriage varied considerably from one country to another. Indeed, while MRSA prevalence in fattening pigs was estimated around 80% in Spain and in The Netherlands, it was estimated at approximately 6% in Switzerland. MRSA has also been recovered from pigs outside Europe, though other methodologies were used (Sergio *et al*., 2007; Khanna *et al*., 2008; Smith *et al*., 2009; Weese and van Duijkeren, 2010; Smith *et al*., 2013). Geographical variations were also found in bovines. Indeed, prevalence ranged from around 1.5% in bulk tank milk from dairy cows in Switzerland (Anon. 2013) to approximately 35 % in Germany (Tenhagen *et al*., 2011). In contrast, bovines being mostly young bulls, tested during a survey performed in Denmark on 192 animals, were all found to be negative for MRSA (DANMAP, 2010). Following these surveys, a technical report aiming at harmonising monitoring and reporting of antimicrobial resistance in MRSA in food-producing animals and food was published (Anon. 2012).

Furthermore, the distribution of MRSA in pigs and bovines seems age dependent. MRSA prevalence in piglets was estimated higher than in sows and fattening pigs, varying between 26% in sows to 41% in piglets (Crombé *et al*., 2012). In bovines, though little information is available on the prevalence of MRSA, similar results have been found. In veal farms in The Netherlands, prevalence was estimated around 28% (Graveland *et al*., 2010).

MRSA prevalence recorded in this study was shown to be lower in dairy farms than in veal calf farms.

In poultry, MRSA carriage was first reported in South Korea though not confirmed by PCR (Lee, 2003). Another study demonstrated the presence of MRSA in broilers at slaughterhouses in The Netherlands (Mulders *et al*., 2010). In Belgium, MRSA have been recovered from broilers occasionally (Nemati *et al*., 2008; Persoons *et al*., 2009; Pletinckx *et al*., 2011). MRSA was rarely found in diseased breeder turkeys in France (Argudín *et al*., 2013) though a German study found 18 out of 20 fattening flocks of turkeys positive for MRSA (Richter *et al*., 2012).

2.2.2. In diseased animals

MRSA has been recovered from sick animals suffering from various infections. In pigs, MRSA was recovered from a case of exudative epidermidis (van Duijkeren *et al*., 2007), from various pathological legions such as arthritis, lungs, limbs and brain lesions or abscesses in the Netherlands (Van der Wolf *et al*., 2012). In Germany, MRSA has also been recovered from urinary-genital tract infections (Schwarz *et al*., 2008). In bovines, MRSA is considered to play an important role in bovine mastitis and has been found in cases of subclinical and clinical mastitis in numerous countries such as Belgium (Vanderhaeghen *et al*., 2010b; Bardiau *et al*., 2013), South Korea (Lee, 2003) and Germany (Feβler *et al*., 2010). Different cases of wound infections assigned to MRSA have also been reported in horses (Hartmann *et al*., 1997; Seguin *et al*., 1999; van Duijkeren *et al*., 2010), dogs (Gortel *et al*., 1999) and wild animals such as hedgehogs (Monecke *et al*., 2013a).

2.3. Virulence in LA-MRSA

S. aureus is considered as an important pathogen that may cause various infections. However, LA-MRSA (mainly belonging to ST398) does not often carry important virulence determinant commonly found in community associated (CA)- or hospital acquired (HA)-MRSA except for the hemolysin-encoding genes that seem to be frequently detected in LA-MRSA (Monecke *et al*. 2007; Kadlec *et al.,* 2009; Feβler *et al*., 2010; Jamrozy *et al*., 2012). Few studies also reported cases of Panton-Valentine leukotoxin (PVL)-positive strains, though these have been reported mostly from humans without animal-contact (van Belkum *et al.* 2008; Yu *et al*., 2008; Stegger *et al*., 2010). Staphylococcal enterotoxins (SEs) have occasionally been reported in MRSA of pigs (Kadlec *et al.,* 2009; Laurent *et al*., 2009; Argudín *et al*., 2011). Other virulence factors commonly considered to be involved in a wide variety of *S. aureus* infections, such as exfoliatins (ET), leukotoxins and Toxic Shock Syndrome Toxin-1 (TSST-1), are rarely found in LA-MRSA strains (Kadlec *et al.,* 2009; Feβler *et al*., 2010; Jamrozy *et al*., 2012).

2.4. Antimicrobial resistance encountered in livestock associated MRSA

2.4.1. Resistance to β-lactams

β-lactam resistance is mainly based on two mechanisms, namely the inactivation of β-lactam antibiotics by β-lactamases and the production of a low-affinity penicillin-binding protein 2a (PBP2a). The production of β-lactamases, which confer only resistance to penicillins (Dyke and Gregory, 1997), is encoded by the *blaZ* gene which is tightly regulated by, *blaI* (inducer) and *blaR* (repressor). This *bla* operon is located on plasmids and/or chromosomally on transposons (Tn)*552*-like (Jensen and Lyon, 2009). The PBP2a is encoded by the *mecA* gene located on the SCC*mec*. This PBP confers resistance to almost all β-lactam antibiotics including methicillin, oxacillin and cephalosporins. Additionally to this *mecA*

gene, a novel *mecA* homologue, called *mecC*, has recently been described. Previously known as $mecA_{LGA251}$, this homologue was first recovered from MRSA isolates from bovines and humans in the UK (García-Álvarez *et al*., 2011).

Several studies reported the presence of the penicillin resistance encoding gene *blaZ* in MRSA from pigs (Argudín *et al*., 2011; Wendlandt *et al*., 2013a), cattle (Feßler *et al*., 2010), horses (Walther *et al*., 2009), sheep (Gharsa *et al*., 2012), chickens and turkeys (Monecke *et al*., 2013b). Moreover, the methicillin resistance encoding gene, *mecA*, was recovered in MRSA isolates from pigs (Argudín *et al*., 2011; Wendlandt *et al*., 2013a), cattle (Vanderhaeghen *et al*., 2010b; Wendlandt *et al*., 2013a), sheep (Feßler *et al*., 2012), poultry (Wendlandt *et al*., 2013a; Argudín *et al*., 2013) and horses (Cuny *et al*., 2008; van Duijkeren *et al*., 2010). Reported resistance genes recovered in LA-MRSA belonging mainly to CC398 are summarized in table 3.

2.4.2. Resistance to tetracyclines

Tetracycline resistance is based on active efflux via Tet(K) or Tet(L) proteins of the Major Facilitator Superfamily or on ribosomal protection via Tet(M) or Tet(O) proteins (Wendlandt *et al*., 2013a). The *tet*(K) and *tet*(L) genes are often located on plasmids and have been identified in staphylococci of animal origin. Additionally, *tet*(M) is also commonly observed in staphylococci of animal origin and is frequently located on a transposon of enterococcal origin. In contrast, *tet*(O) gene has rarely been found in staphylococci (Wendlandt *et al*., 2013a).

The tetracycline resistance gene, *tet*(L) has been detected in LA-MRSA from diseased pigs (Kadlec and Schwarz, 2009a) and bovine mastitis (Feβler *et al*., 2010). Recent studies showed also the presence of the genes *tet*(M), *tet*(K) and tet(L) in various combinations in

LA-MRSA from pigs, cattle, or chickens and ducks (Argudín *et al*., 2011; Wendlandt *et al*., 2013a).

2.4.3. Resistance to aminoglycosides and aminocyclitols

Aminoglycoside resistance is based on enzymatic inactivation. Several genes coding for inactivating enzymes with a variable substrate spectrum have been identified. The gene *aacA-aphD* codes for a bifunctional enzyme that shows acetyltransferase and phosphotransferase activity and confers resistance to gentamicin, kanamycin and tobramycin. The gene *aadD* codes for an adenyltransferase, which confers resistance to kanamycin, neomycin and tobramycin. The gene *aphA3* codes for a phosphotransferase which mediates resistance to kanamycin, neomycin, and amikacin. Finally, the gene *aadE* encodes an adenyltransferase, which confers streptomycin resistance. Most of these genes are plasmid- or transposon-borne (Wendlandt *et al*., 2013a; Wendlandt *et al*., 2013b). Additionally to these aminoglycoside resistance genes, the *str* gene encoding an adenyltransferase mediates streptomycin resistance.

The *aacA-aphD*, *aadD* and *aphA3* genes have been detected in MRSA from different animal origins such as pigs, cattle, horses, chickens and turkeys (Walther *et al*., 2009; Kadlec and Schwarz, 2009b; Feßler *et al*., 2010; Argudín *et al*., 2011; Monecke *et al*., 2013b; Wendlandt *et al*., 2013a). Streptomycin resistance (*str*) encoding gene has been identified in porcine MRSA (Overesch *et al*., 2011).

2.4.4. Resistance to macrolides, lincosamides and streptogramins

Resistance to macrolides, lincosamides and streptogramins can be mediated by a number of different genes that code for either target site modifying enzymes, these antimicrobial agents inactivating enzymes or efflux systems (Wendlandt *et al*., 2013a). The

combined resistance to macrolides, lincosamides and streptogramin B (MLS_B) is encoded by *erm* genes coding for methylases that modify the target site in 23S rRNA. Combined resistance to macrolides and streptogramin B (MS_B) is encoded by the *msr*(A) gene, an ATP binding cassette (ABC) transporter protein. The *mph*(C) and *lnu*(A) encode resistance to macrolides resistance and lincosamides respectively. The *mph*(C) gene encodes a macrolide phosphotransferase and *lnu*(A) encodes a lincosamide nucleotidyltransferase. In addition to the lincosamid resistance gene, the plasmid-borne *lsa*(B) encodes an ABC transporter protein which has been reported to confer decreased susceptibility to lincosamides. Inactivation of streptogramin A and streptogramin B only are respectively due to *vat* (A, B or C) acetyltransferase encoding gene and *vgb*(A and B) coding for streptogramin B lyases.

MLS_B resistance encoding genes of the *erm* family have broadly been detected in MRSA of animal origin. The *erm*(A) gene has been identified in MRSA from pigs, bovines, horses, chickens and turkeys (Wendlandt *et al*., 2013a). The *erm*(B) gene has also been detected in LA-MRSA from pigs (Kadlec *et al*., 2009) and cattle (Feßler *et al*., 2010). The *erm*(C) genes has been detected in various livestock animals such as pigs, bovines, horses, sheep, chickens and turkeys (Wendlandt *et al*., 2013a). Furthermore, *erm*(T), together with other resistance genes, have been recovered from MRSA isolates from pigs, bovines, chickens and turkey (Wendlandt *et al*., 2013a). Additionally to these MLS_B resistance encoding genes, *lnu*(A or B) coding for lincosamide resistance were recovered from dairy cows, turkeys and pigs (Argudín *et al*., 2011; Wendlandt *et al*., 2013a).

2.4.5. Resistance to phenicols

Among staphylococci of animal origin, resistance to non-fluorinated phenicols can be mediated by enzymatic inactivation via chloramphenicol acetyltransferases encoded by the *cat* genes, cat_{pC221}, cat_{pC223} or cat_{pC194}, named according to the plasmids on which they have

been identified. Resistance to fluorinated phenicols is based on either efflux via a phenicol-specific exporter encoded by the gene *fexA* or on target site modification by an rRNA methylase encoded by the gene *cfr* (Wendlandt *et al*., 2013a). The *cfr* gene was first described as a chloramphenicol resistance mechanism in *S. sciuri* (Schwarz *et al*., 2000) though the methylation encoded by this gene leads to a multi-drug resistance phenotype affecting the binding of various antimicrobials including phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (Long *et al*., 2006). This methyltransferase has also been shown to increase the minimal inhibitory concentrations (MICs) of certain 16-membered macrolides, such as spiramycin (Shen *et al*., 2013).

The *fexA* gene has been detected in MRSA from pigs (Argudín *et al*., 2011; Wendlandt *et al*., 2013a), cattle (Feßler *et al*., 2010), and a horse (Kehrenberg and Schwarz, 2006). Pleuromutilin resistance gene belonging to the *vga* (A or C) and *lsa*(C) have also been recovered from MRSA originating from pigs, cattle and turkeys (Wendlandt *et al*., 2013a). The multi-resistance encoding gene, *cfr*, was recovered from porcine and bovine MRSA (Argudín *et al*., 2011; Wendlandt *et al*., 2013a).

Table 3. Reported resistance genes recovered in MRSA in farms animals

Antimicrobial resistance	Gene(s) related	Animal associated	Reference
Penicillins	*blaZ*	Pig	Kadlec *et al*., 2009; Argudín *et al*., 2011; Overesch *et al*., 2011
		Bovine	Feßler *et al*., 2010
		Horse	Walther *et al*., 2009
		Sheep	Gharsa *et al*., 2012
		Poultry	Monecke *et al*., 2013b; Argudín *et al*., 2013
β-Lactams	*mecA*	Pig	Voss *et al*., 2005; de Neeling *et al*., 2007; van Duijkeren *et al*., 2007; Kadlec *et al*., 2009; Wagenaar *et al*., 2009; Argudín *et al*., 2011; Overesch *et al*., 2011
		Bovine	Juhász-Kaszanyitzky *et al*., 2007; Monecke *et al*., 2007; Feßler *et al*., 2010, 2012; Vanderhaeghen *et al*., 2010b; Holmes and Zadoks, 2011; Spohr *et al*., 2011;
		Horse	X.M. Wang *et al*., 2012; Cuny *et al*., 2008; Walther *et al*., 2009; van Duijkeren *et al*., 2010; Sieber *et al*., 2011
		Sheep	Feßler *et al*., 2012; Gharsa *et al*., 2012
		Goat	Chu *et al*., 2012
		Poultry	Nemati *et al*., 2008; Persoons *et al*., 2009; Monecke *et al*., 2013b; Argudín *et al*., 2013
	mecC (*mec*$_{ALGA251}$)	Bovines	García-Álvarez *et al*., 2011
Tetracyclines	*tet*(L)	Pig	Kadlec *et al.,* 2009
		Bovine	Feβler *et al*., 2010
		Poultry	Argudín *et al*., 2011
	tet(M)	Turkeys	Argudín *et al*., 2013
	Combinaison tet(K), tet(L), *tet*(M)	Various animal source	Kadlec *et al*., 2009; Argudín *et al*., 2011
All phenicols	*fexA*	Pig	Kadlec *et al*., 2009; Kehrenberg *et al*., 2009; Argudín *et al*., 2011; Wang *et al*., 2012
		Bovine	Feßler *et al*., 2010
		Horse	Kehrenberg and Schwarz, 2006
Aminoglycosides (gentamicin, kanamycin, tobramycin, amikacin)	*aacA-aphD*	Pig	Schwarz *et al*., 2008; Kadlec *et al*., 2009; Argudín *et al*., 2011; Overesch *et al*., 2011
		Bovine	Turutoglu *et al*., 2009; Feßler *et al*., 2010;
		Horse	Cuny *et al*., 2006; Walther *et al*., 2009; Sieber *et al*., 2011
		Poultry	Monecke *et al*., 2013b
Aminoglycosides (kanamycin, neomycin, tobramycin)	*aadD*	Pig	Kadlec and Schwarz, 2009b; Argudín *et al*., 2011
		Bovine	Feßler *et al*., 2010
		Horse	Walther *et al*., 2009
		Poultry	Monecke *et al*., 2013b

Aminoglycosides (kanamycin, neomycin, amikacin)	*aphA3*	Pig Bovine Horse	Argudín *et al*., 2011 Turutoglu *et al*., 2009; Feßler *et al*., 2010; Walther *et al*., 2009
Aminoglycosides (streptomycin)	*str*	Pig	Overesch *et al*., 2011
Macrolides, lincosamides, streptogramin B (MLS_B)	*erm*(A)	Pig Bovine Horse Poultry	Kadlec *et al*., 2009; Argudín *et al*., 2011 Feßler *et al*., 2010 Walther *et al*., 2009 Monecke *et al*., 2013b
	erm(B)	Pig Bovine	Kadlec *et al*., 2009 Feßler *et al*., 2010
	erm(C)	Pig Bovine Horse Sheep	Kadlec *et al*., 2009; Argudín *et al*., 2011 Feßler *et al*., 2010 Walther *et al*., 2009 Gharsa *et al*., 2012
	erm(T)	Pig Bovine Poultry	Kadlec and Schwarz, 2010a Feßler *et al*., 2010b Monecke *et al*., 2013b
Lincosamides	*lnu*(A)	Bovine Poultry	Argudín *et al*., 2011; Lozano *et al*., 2012 Monecke *et al*., 2013b
	lnu(B)	Pig	Li *et al*., 2013
Lincosamides, pleuromutilins, streptogramin A	*vga*(A)	Pig Bovine Poultry	Kadlec *et al*., 2009, 2012; Overesch *et al*., 2011 Feßler *et al*., 2010 Monecke *et al*., 2013b
	vga(C)	Pig Bovine Pig Bovine Poultry	Kadlec and Schwarz, 2009b; Kadlec *et al*., 2010a Feßler *et al*., 2010 Schwendener and Perreten, 2011 Hauschild *et al*., 2012 Hauschild *et al*., 2012; Monecke *et al*., 2013b
	lsa(C)	Pig	Li *et al*., 2013
All phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	*cfr*	Pig Bovine	Kehrenberg *et al*., 2009; Argudín *et al*., 2011; Wang *et al*., 2012 Wang *et al*., 2012
Trimethoprim	*dfrA* (*dfrS1*)	Pig Horse	Argudín *et al*., 2011 Walther *et al*., 2009
	dfrD	Pig	Argudín *et al*., 2011
	dfrG *dfrK*	Pig Pig Bovine Poultry Horse	Kadlec *et al*., 2009; Argudín *et al*., 2011; Overesch *et al*., 2011 Kadlec *et al*., 2009; Argudín *et al*., 2011 Feßler *et al*., 2010 Monecke *et al*., 2013b Sieber *et al*., 2011
Fusidic acid	*fusB*	Sheep	Gharsa *et al*., 2012

2.4.6. Resistance to trimethoprim

Trimethoprim resistance in animal staphylococci is commonly based on the presence of plasmid- or transposon-borne *dfr* genes (*dfrA*, *dfrD*, *dfrG* or *dfrK*) which code for trimethoprim-insensitive dihydrofolate reductases (Wendlandt *et al*., 2013a). While *dfrA* is widespread among staphylococci of humans, it has rarely been found in animals. *dfrD* is even more rare in staphylococci from animal origin. In contrast, *dfrG* and *dfrK* have been detected in MRSA isolated from several animals including dogs, pigs, chickens and turkeys (Argudín *et al*., 2011; Wendlandt *et al*., 2013a).

2.4.7. Resistance to fusidic acid

Fusidic acid resistance is caused by a mutation in the gene *fusA*, a chromosomal gene encoding the elongation factor G (EF-G), or by the *fusB* gene expressing a Fus protein that protects the drug target. The latter gene was found on a penicillinase carrying plasmid (pUB101) that can also be integrated into the chromosome. Additionally, chromosomal genes, *fusC* and *fusD*, encoding a cytoplasmatic protein, have also been identified and were shown to confer resistance to fusidic acid as well (Lannergård *et al*., 2009).

Fusidic acid resistance encoding gene (*fusB*) is very rare though it has been detected in MRSA isolated from sheep (Gharsa *et al*., 2012).

2.4.8. Resistance to mupirocin

Resistance to mupirocin in staphylococci is commonly due to mutations in the *ilesS* gene or due to a mupirocin-insensitive isoleucyl-tRNA synthase encoded by *ileS2* (also called *mupA*) or *mupB*.

Chapter 3 - The ecological importance of the *Staphylococcus sciuri* species group as a reservoir for resistance and virulence genes

3.1. Introduction

Approximately one century after the description of the genus *Staphylococcus* in 1880, *S. sciuri* was discovered by Kloos *et al*. (1976a) and described as a common bacterium living in a very broad range of habitats. This species was found to be closely related to other species that were comprised in the *S. sciuri* species group. This group is now composed of coagulase-negative and novobiocin-resistant bacteria and includes *S. sciuri*, *S. lentus, S. vitulinus, S. fleurettii* and *S. stepanovicii* (De Vos *et al*., 2009, Hauschild *et al*., 2010). Those five species are mainly considered as commensal animal-associated species though sometimes also recovered from dust and the environment (Kloos *et al*., 1976a). While other staphylococcal species such as *S. aureus* are well known for their clinical importance (Lowy, 1998), members of this group are mainly recovered from healthy animals (Kawano *et al*., 1996; Stepanović *et al*., 2001a; Yasuda *et al*., 2002). However, the *S. sciuri* species group has an interesting feature since members of this group are known to carry different homologues of the methicillin resistance gene *mecA* in their chromosomal DNA. Nevertheless, these homologues do not confer methicillin resistance (Monecke *et al*., 2012) as does the *mecA* gene that is located on the SCC*mec* (Ito and Hiramatsu, 1998). Members of the *S. sciuri* species group have occasionally been found in clinical infections in animals (Frey *et al*., 2013) and humans (Stepanović *et al*., 2003).

3.2. Characteristics of the *Staphylococcus sciuri* species group and its position in the genus *Staphylococcus*

S. sciuri is considered as one of the most primitive species within the genus *Staphylococcus* and was first described by Kloos *et al*. (1976a) when strains were isolated from the skin of animals and humans. The *S. sciuri* species group belongs – together with the *Staphylococcus saprophyticus* group (*S. saprophyticus*, *Staphylococcus cohnii* and *Staphylococcus xylosus*) – to the novobiocin-resistant CoNS. The members of the *S. sciuri* group are oxidase-positive and their cell wall is characterized by its peptidoglycan type Lys-Ala-Gly_4 (De Vos *et al*., 2009). *S. sciuri* was first divided in the two subspecies *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *lentus* (Kloos *et al*., 1976a). However, based on DNA-DNA hybridization studies and re-examination of physiological characteristics such as their peptidoglycan type and oxidase reaction, Schleifer *et al*. (1983) reclassified *S. sciuri* subsp. *lentus* as *S. lentus*. *S. sciuri*, however, was again divided in three subspecies on the basis of their ribotype patterns (Kloos *et al*., 1997). These subspecies were called *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium*. Later on, another species named *Staphylococcus vitulus* was found to be closely related to *S. sciuri* and *S. lentus* using DNA-DNA hybridization and was described as a third species belonging to the *S. sciuri* species group (Webster *et al*., 1994). This name was corrected four years later to *S. vitulinus* (Trüper and De'Clari, 1998). In 1995, a fourth novobiocin-resistant and oxidase-positive species named *Staphylococcus pulvereri* was described by Zakrzewska-Cerwińska *et al*. (1995). However, DNA-DNA hybridization showed that this species and *S. vitulinus* were so closely related that it was proposed to consider *S. pulvereri* as synonym of *S. vitulinus* (Švec *et al*., 2004). In 2000, Vernozy-Rozand *et al.* (2000) described a new oxidase-positive species isolated from goat milk cheese. This new species was named *S. fleurettii* and is now considered as the fourth member of the *S. sciuri* species group. More recently, a fifth species

was recovered from the skin, fur and intestinal tracts of free-living small mammals (rodents and insectivores). This species was called *S. stepanovicii*, in honour of Serbian microbiologist Srdjan Stepanović, for his contributions to the study of members of the *S. sciuri* group (Hauschild *et al*., 2010). Main characteristics differentiating these species and subspecies are shown in table 4 (De Vos *et al*., 2009; Hauschild *et al*., 2010). This group has a feature with its ubiquitous presence of *mecA* homologues which have approximately 80% nucleotide sequence identity to the *mecA* carried by MRSA (Wu *et al*., 1996; Wu *et al*., 1998, Monecke *et al*., 2012). These *mecA* homologues found in *S. sciuri*, *S. vitulinus* and *S. fleurettii* were shown not to be associated with SCC*mec* and located in the chromosomal DNA linked to essential genes for the growth of staphylococci (Tsubakishita *et al*., 2010). However, the species-specific *mecA* homologues from *S. sciuri* and its subspecies did not confer clinical resistance to methicillin (Yasuda *et al*., 2002, Monecke *et al*., 2012). The *mecA*-carrying *S. vitulinus* were found to be susceptible to penicillin *in vitro* (Schnellmann *et al*., 2006). To date, it seems that *S. fleurettii* contains the common ancestor of the other *mecA* genes in the *S. sciuri* species group and to be the ancestor of the acquired *mecA* gene conferring clinical methicillin resistance in other staphylococci, including species of the *S. sciuri* species group. Indeed, the *S. fleurettii* homologue was shown to have 99% to 100% sequence homology with the *mecA* present in MRSA strain N315 and strains carrying SCC*mec* types II, III and VIII (Tsubakishita *et al*., 2010). Additionally to this, the presence of non-typeable SCC*mec* in CoNS including *S. sciuri*, *S. lentus* and *S. fleurettii* indicates the presence of novel SCC*mec* elements (Tulinski *et al*., 2012).

Table 4. Main characteristics differentiating species and subspecies of the *Staphylococcus sciuri* species group.

Characteristics	*S. sciuri* subsp. *sciuri*	*S. sciuri* subsp. *carnaticus*	*S. sciuri* subsp. *rodentium*	*S. lentus*	*S. vitulinus*	*S. fleurettii*	*S. stepanovicii*
Colony size > 6mm	+	-	+	-	-	-	+
Clumping factor	-	d	+	-	d	-	-
Activity of :							
Urease	-	-	-	-	-	-	+
DNase	+	ND	ND	+w	d	+w	+
Alkaline phosphatase	d	d	d	w	-	-	-
Acid production from:							
l-arabinose	d	d	d	d	-	d	-
d-cellobiose	+	d	d	+	(d)	-	-
Lactose	d	d	-	d	-	-	-
Maltose	(d)	(+)	(+)	d	-	+	+w
d-mannitol	+	+	+	+	+	-	+
d-mannose	(d)	d	+	(+)	-	+	+
Raffinose	-	-	-	+	-	-	-
d-ribose	+	+	+	+	d	-	-
d-trehalose	+	+	+	+	(d)	+	+
d-turanose	-	-	-	-	-	+	-

Symbols: +, 90% or more strains positive; -, 90% or more stains negative; d, 11-89% strains positive; ( ) delayed reaction; w, weak reaction; +w, positive to weak reaction; ND, not determined.

3.3. Epidemiology

The members of the *S. sciuri* species group are considered as very common bacteria that are recovered from a broad range of hosts and the environment (Kloos *et al.*, 1976a). Moreover, dust containing *S. sciuri* could be the vehicle for dispersal of this bacterium, as has been suggested in studies in military barracks and hospitals (Couto *et al*., 2000; Dakić *et al*., 2005). In fact, it is well known that staphylococci withstand well desiccation and are likewise frequently isolated from hospital dust (Dancer, 1999; Wagenvoort *et al*., 2000). Furthermore, it has been reported that *S. sciuri* may be capable of a free-living existence (Kloos, 1980).

Only few researchers have been looking at the presence of *S. sciuri* in humans. *S. sciuri* has been isolated from nares of healthy human carriers in Indonesia (Severin *et al*., 2010) and France (Marsou *et al*., 1999), nares and axillae of healthy human carriers in Portugal (Couto *et al*., 2000) and vagina among humans in Morocco (Marsou *et al*., 1999) and Czech Republic (Stepanović *et al*., 2005a). Furthermore, despite their role as commensal bacteria, members of the *S. sciuri* species group may occasionally cause disease in humans and other hosts (Adegoke, 1986).

3.3.1. Healthy animals and food

S. sciuri and its subspecies have been recovered from a very broad range of warm blooded animals. Ever since *S. sciuri* has been isolated from squirrels (as the name refers to *Sciurus*, the generic name for squirrel), it subsequently has been recovered from a wide variety of wild animals including marsupials, rodents, carnivores, monkeys, cetaceans and domestic animals such as cattle, sheep, horses and dogs (Kloos *et al*., 1976b; Adegoke, 1986; Kawano *et al*., 1996; Stepanović *et al*., 2001a; Yasuda *et al*., 2002). *S. sciuri* subsp. *carnaticus* (whose name pertains to meat) was recovered mostly from cattle but also from dolphins and South American rodents of the species of acouchis (Kloos *et al*., 1997). As its

name says, *S. sciuri* subsp. *rodentium* has mainly been recovered from rats and squirrels but nevertheless also from whales (Kloos *et al*., 1997). The current subdivision in subspecies refers to a certain host preference, though the host can be quite diverse. One should of course be conscious that there might be still big gaps in the knowledge on the prevalence of the different subspecies since they have only been studied scarcely.

S. lentus, which was named as such because of its slow growth, has been recovered from different domestic animals including poultry, pigs, cattle, goats, sheep and horses (Schleifer *et al*., 1983; Devriese *et al*., 1985; Busscher *et al*., 2006). *S. lentus* and *S. sciuri* have also both been isolated from animal-derived products such as meat and bovine or goat milk (Deinhofer and Pernthaner, 1995; Huber *et al*., 2011; Bhargava and Zhang, 2012).

Since *S. vitulinus* had its name corrected in 1998, epidemiological data also have to be found in papers using its former denomination, *S. vitulus*. This species has been isolated from horses (Bagcigil *et al*., 2007; Moodley and Guardabassi, 2009; Karakulska *et al*., 2012), poultry (Webster *et al*., 1994) and from frozen food samples in Korea (Baek *et al*., 2009).

Being the last species described of the *S. sciuri* species group, *S. fleurettii* and *S. stepanovicii* have not often been identified so far. To date, *S. fleurettii* has been isolated form goat milk (Vernozy-Rozand *et al*., 2000), cats, chicken, horses (Tsubakishita *et al*., 2010), pigs (Vanderhaeghen *et al*., 2012b), cows and minced meat (Huber *et al*., 2011). *S. stepanovicii* has been recovered from free living rodents and insectivores (Hauschild *et al*., 2010)

Most studies focused on the prevalence of CoNS as a whole group and studies on prevalence at the species level are presented only as a proportion of CoNS isolated. Nevertheless, *S. sciuri* species group members have been shown to be the most abundant species among the CoNS encountered in different studies such as in healthy horses (Busscher

et al., 2006; Moodley and Guardabassi, 2009) and other farm animals (Devriese *et al*., 1985; Huber *et al*., 2011; Bhargava and Zhang, 2012).

3.3.2. Diseased animals

Members of the *S. sciuri* species group have also been isolated from sick animals. Indeed, *S. sciuri* and *S. fleurettii* have been recovered from several cases of bovine mastitis (Rahman *et al*., 2005; Lüthje and Schwarz, 2006; Nam *et al*., 2010; Frey *et al*., 2013). *S. sciuri* has also been isolated from sick goats suffering of ovine rinderpest (Adegoke, 1986), canine dermatitis (Hauschild and Wójcik, 2007) and an outbreak of fatal exudative epidermitis in piglets in China (Chen *et al*., 2007). *S. lentus* has been isolated from sick goats and poultry (Adegoke, 1986).

We could find very few reports on *S. fleurettii* and *S. vitulinus* isolated from diseased animals. They have only been implicated in clinical and subclinical cases of bovine mastitis (Frey *et al*., 2013). While CoNS are considered in some countries as the most common mastitis agents (Pitkälä *et al*., 2004; Taponen *et al*., 2006), this implies staphylococcal species other than members of the *S. sciuri* group such as *Staphylococcus chromogenes*, *Staphylococcud simulans* and *Staphylococcus epidermidis* which were often found to be the most abundant species (Lüthje and Schwarz, 2006; Santos *et al*., 2008; Persson Waller *et al*., 2011). It should be noted however that the pathogenic role of CoNS in mastitis is much debated, and some investigators suggest it is merely a contaminant (Huebner and Goldmann, 1999).

3.4. Population structure

3.4.1. Identification of the species of the S. sciuri group

The identification of bacteria from the *S. sciuri* group was initially based on phenotypic characteristics (Kloos and Schleifer, 1975; Bannerman, 2003). Currently, some studies still use commercial kits based on the biochemical profiles, but these kits have been shown to have low accuracy (Heikens *et al*., 2005; Zadoks and Watts, 2009; Geraghty *et al*., 2013). In fact, misidentification of the members of the *S. sciuri* species group by commercial identification systems has been reported on several occasions (Skulnick *et al*., 1989; Matthews *et al*., 1990; Stepanović *et al*., 2005b). Several more accurate genotypic methods have been developed for species-level identification of the *S. sicuri* group bacteria, including methods based on species-specific primers, the determination of species-specific gene sequences, analysis of length polymorphism of the intergenic spacers between transfer (t)RNA genes (tRNA-intergenic spacer PCR or tDNA-PCR) associated with capillary electrophoresis, or ribotyping (Gribaldo *et al*., 1997; Kloos *et al*., 1997; Mendoza *et al*., 1998; Baele *et al*., 2000; Couto *et al*., 2001; Lee and Park, 2001; Poyart *et al*., 2001; Shittu *et al*., 2004; Becker *et al*., 2005; Shah *et al*., 2007; Supré *et al*., 2009; Blaiotta *et al*., 2010; Park *et al*., 2010; Sasaki *et al*., 2010; Hwang *et al*., 2011). Some studies have evaluated different typing techniques for identification of staphylococci (Zadoks and Watts, 2009; Geraghty *et al*., 2013). Currently the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) seems to be the most suitable method for the indentification of the species within the *S. sciuri* species group (Carbonnelle *et al*., 2007; Bergeron *et al*., 2010; Dubois *et al*., 2010; Loonen *et al*., 2012). Unfortunately, none of these methods is 100% correct and a combination of different methods is necessary to accurately identify these bacteria. Therefore, many studies did not further identify, or have uncertain identifications,

making it sometimes difficult to interpret the true population structure of the different species of the *S. sciuri* species group.

3.4.2. Intra-species variability

The clonal relatedness between isolates of the *S. sciuri* species group has mainly been assessed by macrorestriction analysis followed by PFGE. This methodology has been proved a sensitive technique for epidemiological investigation of the clonal relatedness of other staphylococcal species (Murchan *et al*., 2003, Miragaia *et al*., 2008). The subtyping analyses of strains of the *S. sciuri* species group have been performed using diverse *S. aureus* protocols (de Lencastre *et al*., 1994; Bannerman *et al*., 1995; Mulvey *et al*., 2001; Murchan *et al*., 2003; McDougal *et al*., 2003). Numerous studies have reported the existence of equal or similar PFGE profiles within each *S. sciuri* (Couto *et al*., 2000; Hauschild and Schwarz, 2003; Moodley and Guardabassi, 2009; Aslantaş *et al*., 2012), *S. lentus* (Zhang *et al*., 2009; Aslantaş *et al*., 2012) or *S. vitulinus* (Moodley and Guardabassi, 2009) population investigated. Some of these studies have demonstrated that the main sources of human and animal colonization may be the environmental niche (Couto *et al*., 2000; Hauschild and Schwarz, 2003; Dakić *et al*., 2005; Moodley and Guardabassi, 2009; Zhang *et al*., 2009; Aslantaş *et al*., 2012). Unfortunately, the different studies used different PFGE typing protocols, and thus do not allow a proper inter-study comparison. Further studies using other typing techniques, with more phylogenetic information, and the development of a harmonized PFGE protocol for the *S. sciuri* species group are necessary to better understand the clonality and population structure of these bacteria.

3.5. Virulence

Although the members of the *S. sciuri* species group have been shown to be facultative pathogens that may cause invasive disease in animals and humans, the possible virulence factors of these bacteria have not been intensively studied. Few studies have shown that *S. sciuri* strains may possess a wide spectrum of virulence factors (Table 5). Some virulence factors displayed (lipolytic, proteolytic or hemolytic) activities similar to those of other staphylococci involved in pathogenic processes such as *S. aureus* (Stepanović *et al*., 2001b). Other virulence factors are typically related to other staphylococci such as enterotoxins (of *S. aureus*) or the exfoliative toxin C (of *S. hyicus*) (Table 5). In contrast to the factors displayed in Table 5, it has been reported that *S. sciuri* do not have lecithinase, fibrinolysin, urease and starch hydrolysis activity (Stepanović *et al*., 2001b). Additionally, members of the *S. sciuri* species group have been reported susceptible to the activity of lysozyme (Bera *et al*., 2006). These studies underline, that members of the *S. sciuri* species group could acquire diverse virulence factors from other staphylococci through horizontal gene transfer that could further strengthen the pathogenic potential of these bacteria.

Table 5. Virulence factors of the *S. sciuri* species group

Virulence factor	Gene(s) related	Species and subspecies associated	Reference
Biofilm formation	NS	*S. sciuri*	Stepanović *et al*., 2001b; Garza-González *et al*., 2010
	icaA	*S. sciuri*	Rumi *et al*., 2013
Clumping factor	NS	*S. sciuri*	Stepanović *et al*., 2001b
DNase activity	NS	*S. sciuri*	Stepanović *et al*., 2001b
δ hemolysin	NS	*S. sciuri*	Stepanović *et al*., 2001b
Enterotoxins	NS	*S. sciuri; S. lentus*	Valle *et al*., 1990; Vernozy-Rozand *et al*., 1996
	seb	*Scs*. *carnaticus*	Park *et al*., 2011
	sec	*S. lentus*	Ünal and Çinar, 2012
	sei	*Scs*. *carnaticus*	Park *et al*., 2011
	selj	*S. lentus*	Ünal and Çinar, 2012
	selk	*Scs*. *carnaticus*	Park *et al*., 2011
	seln	*Scs*. *carnaticus*	Park *et al*., 2011
	selq	*Scs*. *carnaticus*	Park *et al*., 2011
Exfoliative toxin C[a]	*exhC*	*S. sciuri*	Li *et al*., 2011a; Li *et al*., 2011b
Lipolytic activity[b]	NS	*S. sciuri*	Stepanović *et al*., 2001b; Devriese *et al*., 1985
Nitric oxide production	NS	*S. sciuri*	Stepanović *et al*., 2001b
Proteolytic activity	NS	*S. sciuri*	Stepanović *et al*., 2001b
Toxic shock syndrome toxin-1	NS	*S. sciuri*	Orden *et al*., 1992

NS, not specified in the study; *Scs*, *S. sciuri* subspecies.

[a]The authors have suggested that the *S. sciuri* strain investigated had acquired the *exhC* gene through horizontal gene transfer from other *exh*-carrying staphylococci, such as *S. hyicus*, the most common agent of exudative epidermitis in piglets (Li *et al*., 2011a; Li *et al*., 2011b).

[b]Discrepant results have been obtained regarding to the lipolytic activity. *S. sciuri* does not exhibit lipolytic activity in the study of Kloos *et al*. (1997). Other studies showed that some *S. sciuri* strains were capable of degrading Tween 20, Tween 40 and tributyrin, but not Tween 80 and Difco lipase reagent (Devriese *et al*., 1985; Stepanović *et al*., 2001b). These discrepant results could be explained by the substrate specificity of staphylococcal lipases or due to differences between the *S. sciuri* strains analysed in each study.

3.6. Antimicrobial resistance

The analysis of the prevalence of resistance against antimicrobial agents by members of the *S. sciuri* species group has been hampered by the fact that frequently the investigated bacteria were not identified at the species or subspecies level. New identification methods allowing easier, more accurate, faster and cheaper identification such as MALDI-TOF will allow more accurate identification and likewise, more detailed studies on the prevalence of resistance in the *S. sciuri* species group will become available (Moser *et al*., 2013).

In this section, we will first discuss the different cases of resistance reported in the species of the *S. sciuri* species group, followed by the resistance genes encoding these resistance properties. This will provide a better overview on the possible role of the members of the *S. sciuri* species group as a reservoir of antimicrobial resistance genes. As shown in figure 2, members of the *S. sciuri* species group share many antimicrobial resistance genes with other CoNS as well as coagulase-positive and -variable staphylococci. Indeed, it is a point of concern that the data on the population structure indicates low host specificity, making the *S. sciuri* species group prone to be efficient donors and recipients for the dispersion of genes between different ecosystems.

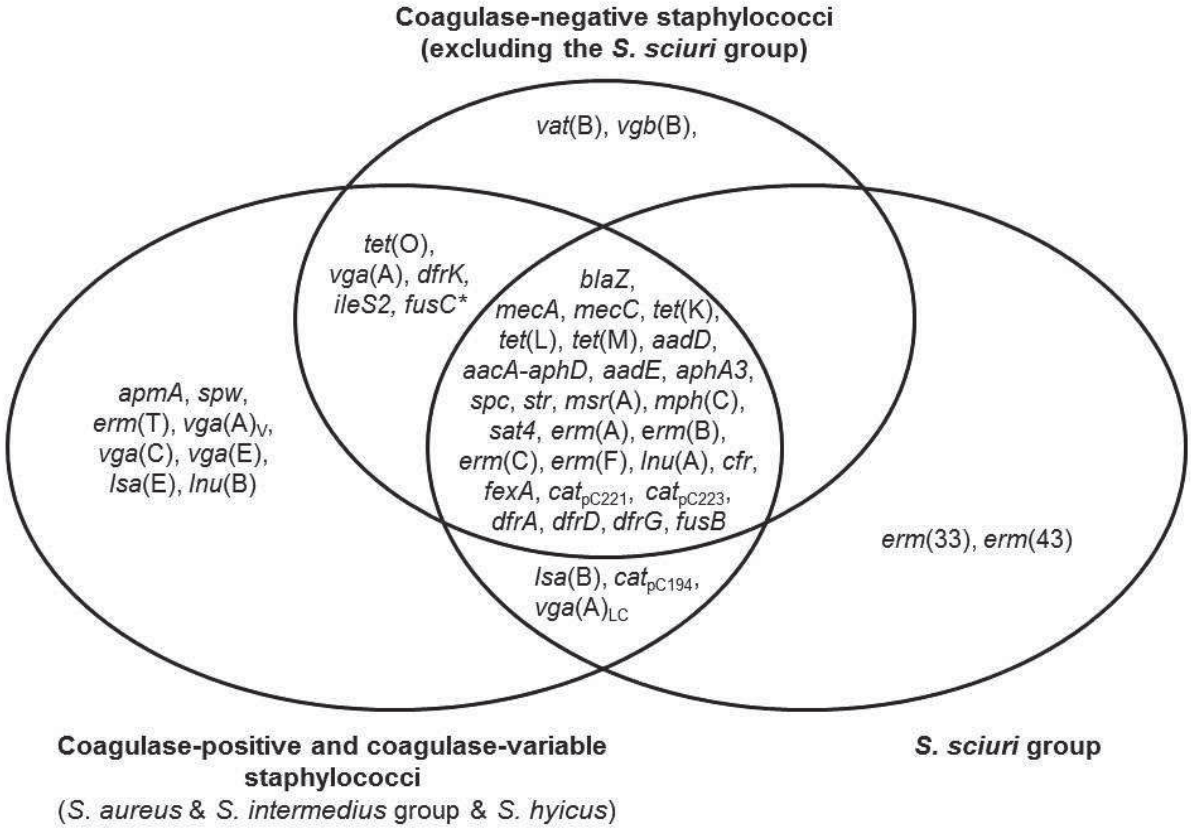

Figure 3. Antimicrobial resistance genes present in coagulase-positive and coagulase-variable staphylococci (left), in coagulase-negative staphylococci (excluding the *S. sciuri* species group) (top) and in members of the *S. sciuri* species group (right) (modified from Wendlandt *et al*., 2013a). Please see the text for the function of the various resistance genes and their presence in the different members of the *S. sciuri* species group. The asterisk (*) indicates the presence of the *fusC* gene in not further specified CoNS (Castanheira *et al*., 2010).

3.6.1. Resistance to β-lactams

Several studies report the presence of methicillin-resistant *S. lentus*, *S. sciuri* and *S. fleurettii*, *S. vitulinus* in horses, horse caretakers, dog and domestic animals as well as on environmental surfaces at farm or in equine hospital (Bagcigil *et al*., 2007; Aslantaş *et al*., 2012) indicating a high degree of colonization. In CoNs, SCC*mec* type III and a non-typeable

SCC*mec* variant were shown to be very common in the *S. sciuri* species group (Damborg *et al*., 2009; Vanderhaeghen *et al*., 2013). Nevertheless, other studies showed also the presence of type II SCC*mec* elements in members of the *S. sciuri* group (Aslantaş *et al*., 2012). Few studies have assessed the presence of resistance to β-lactams in *S. sciuri*, but it was shown to be very high in a Polish hospital (Dakić *et al*., 2005), in which all but one of these strains were also oxacillin resistant.

As discussed above, members of the *S. sciuri* species group have been shown to carry a *mecA* gene that does not confer resistance to β-lactams. For this reason, *S. sciuri* are often considered as methicillin-susceptible (Couto *et al*., 1996). Additionally to the typical *mecA* gene, the newly described homologue, *mecC*, was also found in *S. sciuri* subsp. *carnaticus* isolates cultured from skin infection in cattle (Harrison *et al*., 2013).

3.6.2. Resistance to tetracyclines

Tetracycline resistance seems to vary largely between studies and between the species of the *S. sciuri* species group. In a study from Switzerland, a large number of *S. sciuri* from pigs, cattle, poultry, bulk tank milk, minced meat and abattoir employees were resistant to tetracyclines, while few *S. fleurettii* from pigs, cows, bulk tank milk, minced meat, veterinarians, farmers and abattoir employees, and *S. lentus* from poultry and abattoir employees were tetracycline resistant (Huber *et al*., 2011). A recent study on methicillin-resistant CoNS from veal calves, dairy cows and beef cattle in Belgium identified tetracycline resistance in *S. sciuri* and *S. lentus* but not in *S. fleurettii* isolates (Vanderhaeghen *et al*., 2013). In accordance with this study, Bhargava and Zhang (2012) found tetracycline resistance in *S. lentus* and *S. sciuri* in various farm animals. Additionally, tetracycline resistant *S. sciuri*, *S. lentus* and *S. vitulinus* isolates were found in humans, animals, food, nonhospital and hospital environment between 1998 and 2004 (Hauschild *et al*., 2007a).

The three tetracycline resistance genes, *tet*(K), *tet*(L) or *tet*(M), were carried by at least one isolate of the *S. sciuri* species group (Hauschild *et al*., 2007a). In the same study, the gene *tet*(K) was present in *S. sciuri* subsp. *rodentium* isolates and tetracycline resistant *S. lentus* isolates. The gene *tet*(L) was detected in *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *rodentium* isolates, whereas the gene *tet*(M) was found in *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *rodentium* isolates. The simultaneous presence of *tet*(K)+*tet*(M) and *tet*(L)+*tet*(M) was seen in *S. sciuri* subsp. *rodentium* isolates. A survey on *tet* genes in staphylococci from turkey, ducks, horses, rabbits and guinea pigs (Schwarz *et al*., 1998) showed similar results. The 4.7-kb plasmid pSTS5 that carried a *tet*(K) gene was identified in a *S. sciuri* from a calf and the 4.3-kb plasmid pSTS9 that harbored a *tet*(L) gene was detected in a *S. sciuri* from a pig (Schwarz and Noble, 1994). A *tet*(K) gene was also found together with an *erm*(C) gene on the 6.9-kb plasmid pSTE2 of a *S. lentus* isolate from an insectivore (Hauschild *et al*., 2005). The genes *tet*(K) and/or *tet*(M) were detected in *S. sciuri* from cattle, goats, turkeys, and ducks as well as in *S. lentus* from cattle, goats, sheep, pigs, chickens, ducks, and turkeys in the USA (Bhargava and Zhang, 2012). A study on the antimicrobial resistance of coagulase-negative staphylococci from bovine milk conducted in Switzerland identified the gene *tet*(K) in *S. sciuri*, *S. fleurettii* and *S. vitulinus* (Frey *et al*., 2013).

3.6.3. Resistance to aminoglycosides and aminocyclitols

Resistance to aminoglycosides has been shown to be low in most cases. In general, gentamicin resistance is also lower than kanamycin resistance. A large-scale study by Hauschild *et al*. (2007b) on 304 *S. sciuri* species group isolates revealed a low number isolates resistant to aminoglycosides (gentamicin and kanamycin). Resistance to gentamicin and kanamycin was also detected in *S. lentus* and *S. sciuri* from Belgian cattle, whereas in this

study, all *S. fleurettii* isolates were susceptible to gentamicin and kanamycin (Vanderhaeghen *et al*., 2013).

In the study by Hauschild *et al* (2007b), the genes *aacA-aphD* [also known as *aac(6')-Ie/aph(2'')*; resistance to gentamicin, kanamycin, tobramycin, amikacin], *aadD* [also known as *ant(4')-Ia*; resistance to kanamycin, neomycin, tobramycin], and *aphA3* [also known as *aph(3')-IIIa*; resistance to kanamycin, neomycin, amikacin] either alone or in combination, were found in isolates showing resistance to non-streptomycin aminoglycosides. Among isolates that exhibited resistance to streptomycin, the genes *str* and *aadE* [also known as *ant(6)-Ia*] were identified. Except a single *S. lentus* isolate that was resistant to streptomycin and carried the gene *str*, all other aminoglycoside-resistant isolates were *S. sciuri* (Hauschild *et al*., 2007b). An *aacA-aphD* gene was described on the 43-kb plasmid pGTK2 from *S. sciuri* of chicken origin (Lange *et al*., 2003). In this plasmid, the terminal IS*256* elements of the *aacA-aphD*-bearing transposon Tn*4001* were truncated by the integration of IS*257* elements. The genes *aacA-aphD* and *aadD* have recently been identified on multiresistance plasmids in *S. sciuri* and *S. lentus* from chickens (He *et al*., 2013). Moreover, the gene *aacA-aphD* was also detected on a different type of multiresistance plasmid in a *S. sciuri* from a pig (He *et al*., 2013). An *aacA-aphD* gene was also detected in single *S. sciuri* and *S. fleurettii* isolates from bovine milk (Frey *et al*., 2013).

The 5.1-kb plasmid pSCS12 from a bovine *S. sciuri* isolate was shown to confer resistance to chloramphenicol and streptomycin. Structural analysis showed that this plasmid was an *in-vivo* recombination product of a small pC221-like chloramphenicol resistance plasmid and a small pS194 streptomycin resistance plasmid (Schwarz and Grölz-Krug, 1991). The gene *str* has also been detected recently in *S. sciuri*, *S. fleurettii* and *S. vitulinus* from bovine milk in Switzerland (Frey *et al*., 2013).

In contrast to aminoglycoside resistance, very little information about resistance to aminocyclitols and the presence of the resistance genes *spc* and *spw* (spectinomycin resistance) and *apmA* (apramycin resistance) is currently available (Wendlandt *et al*., 2013a; Wendlandt *et al*., 2013b). The gene *spc* has been identified on the 17.1-kb multi-resistance plasmid pSCFS1 from a bovine *S. sciuri* (Kehrenberg *et al*., 2004). This gene was part of a largely truncated transposon Tn*554* remnant.

3.6.4. Resistance to macrolides, lincosamides and streptogramins

Resistance to erythromycin and clindamycin has been reported in *S. lentus*, *S. sciuri*, and in *S. fleurettii* isolates. Resistance to the streptogramin combination quinupristin/dalfopristin was detected in *S. lentus*, *S. sciuri* and in a single *S. fleurettii* isolate from Belgian cattle (Vanderhaeghen *et al*., 2013).

In their study, Stepanović *et al*. (2006) performed PCR detection of the resistance genes *erm*(A), *erm*(B), *erm*(C) [coding for rRNA methylases that confer combined resistance to MLS_B], *mef*(A) (coding for an efflux protein that confers macrolide resistance), *lnu*(A), and *lnu*(B) (coding for lincosamide-inactivating enzymes). Resistance to macrolides was detected in 10 isolates and two isolates harbored the resistance genes *erm*(B) or *erm*(C). Resistance mediated by active efflux was detected in one isolate. All isolates were susceptible to the streptogramin pristinamycin. The *lnu*(A) gene was detected in two isolates (Stepanović *et al*., 2006). In another study, the genes *erm*(A), *erm*(B) and *erm*(C) alone or in various combinations were detected in *S. sciuri* from cattle, goats, sheep, pigs and turkeys as well as in *S. lentus* from cattle, goats, sheep, pigs, and chickens (Bhargava and Zhang, 2012). An *erm*(B) gene has also been detected in a *S. fleurettii* isolate from bovine milk (Frey *et al*., 2013). An 8-kb plasmid pSES20 from a *S. lentus* of mink origin was found to carry an *erm*(B) gene (Werckenthin *et al*., 1996). This plasmid harbored part of a Tn*917*-like transposon

including the left terminal repeat, the *erm*(B) gene and its regulatory region, as well as the internal direct repeat. A complete Tn*917*-like transposon including the *erm*(B) gene was detected on the 16.4-kb *cfr*-carrying plasmid pBS-01 from porcine *S. sciuri* (Wang *et al*., 2012). More recently, the *erm*(B) gene was detected on different multi-resistance plasmids in *S. sciuri* isolates from pigs and chickens in China (He *et al*., 2013).

Another interesting plasmid is the 6.9-kb plasmid pSTE2 detected in a *S. lentus* from a common shrew (*Sorex araneus*) (Hauschild *et al*., 2005). This plasmid represents the product of an *in vivo* derived RS_A-mediated recombination between two compatible plasmids, a pT181-analogous *tet*(K)-carrying tetracycline resistance plasmid and a pPV141-related *erm*(C)-carrying MLS_B resistance plasmid. An *erm*(C) gene on a small plasmid of 3 kb was also found in a *S. sciuri* from a milk sample of a lactating cow (Khan *et al*., 2000). During the analysis of CoNS from pigs, the 7.1-kb plasmid pSS-03, which harbored the multi-resistance gene *cfr* together with *erm*(C), was identified in four *S. sciuri* isolates (Wang *et al*., 2012). The *erm*(C) gene was also detected on a larger plasmid – again together with *cfr* - in a *S. sciuri* isolate from a chicken (He *et al*., 2013).

The MLS_B resistance gene *erm*(33), so far exclusively detected on the multiresistance plasmid pSCFS1 from bovine *S. sciuri*, represents an *in-vivo* derived product of a recombination between an *erm*(C) gene and an *erm*(A) gene (Schwarz *et al*., 2002). The gene *erm*(F) was detected in *S. lentus* and *S. sciuri* of animal origin (Chung *et al*., 1999). Another novel *erm* gene, *erm*(43), has recently been detected being integrated at the same location in the chromosome in several *S. lentus* isolates of human, dog, and chicken origin (Schwendener and Perreten, 2012).

A *lnu*(A) gene was identified on a plasmid indistinguishable from pLNU1 (Lüthje *et al*., 2007) in a methicillin-resistant *S. sciuri* from the nasal cavity of a pig (Lozano *et al*., 2012). It should also be noted that the ABC transporter gene *lsa*(B), which was reported to elevate the

MICs for lincosamides, was detected on the multiresistance plasmid pSCFS1 from *S. sciuri* (Kehrenberg *et al*., 2004). A variant of the *vga*(A) gene – designated *vga*(A)$_{LC}$ – coding for resistance to lincosamides, pleuromutilins and streptogramin A antibiotics was identified in *S. lentus* from chickens and sheep (Bhargava and Zhang, 2012).

The gene *msr*(A) coding for an ABC transporter that confers resistance to 14-membered MS$_B$ antibiotics was detected in a single *S. vitulinus* isolate from free-living rodents. *S. lentus* and *S. sciuri* isolates from the same sources showed the presence of complete but functionally inactive *mph*(C) genes (Hauschild and Schwarz, 2010).

3.6.5. Resistance to phenicols

In a large scale study, 317 *S. sciuri* species group isolates were investigated for chloramphenicol resistance and the presence of the respective resistance genes. In this study, three *S. sciuri* and one *S. lentus* were found to be chloramphenicol resistant (Hauschild *et al*., 2009).

Plasmids carrying a *cat*$_{pC221}$ gene as the sole resistance gene and differing in their sizes between 2.9 – 4.65 kb were detected in *S. sciuri* isolates from equine (Schwarz *et al*., 1990) and bovine origin (Schwarz and Blobel, 1993). A 5.1-kb plasmid that carried a *cat*$_{pC221}$ gene and a pS194-associated *str* gene for streptomycin resistance was identified in a *S. sciuri* isolate from a calf (Schwarz and Grölz-Krug, 1991). Plasmids of 3.6 – 4.6 kb harbouring *cat*$_{pC221}$ genes as well as plasmids of 4.6 kb which carry the *cat*$_{pC223}$ have been detected in *S. lentus* isolates from mink (Schwarz, 1994). In Hauschild *et al*. (2009), *cat*$_{pC221}$ genes were found in two *S. sciuri* and the single *S. lentus*, a *cat*$_{pC194}$ gene was identified on the 2.9-kb plasmid pSCS34 (Hauschild *et al*., 2009). A *cat*$_{pC221}$ gene was also detected in a single *S. sciuri* isolate from bovine milk (Frey *et al*., 2013)

The first phenicol-specific exporter gene, designated *fexA*, was identified during the analysis of plasmid pSCFS2 from a bovine *S. lentus* isolate (Kehrenberg and Schwarz, 2004). The gene *fexA* codes for a protein of 475 amino acids with 14 transmembrane domains, which differs from all previously known proteins involved in the efflux of chloramphenicol and florfenicol. Induction of *fexA* expression by chloramphenicol and florfenicol occurs via translational attenuation. The gene *fexA* is part of transposon Tn*558* (Kehrenberg and Schwarz, 2005). The *fexA* gene (in part together with the gene *cfr*) has been detected in *S. lentus* and a single *S. sciuri* from pigs and cattle in Germany (Kehrenberg and Schwarz, 2006), in *S. sciuri* from pigs of different farms in China (Wang *et al*., 2012; He *et al*., 2013), but also in *S. sciuri* and *S. lentus* from chickens as well as in a *S. sciuri* from a duck (He *et al*., 2013).

3.6.6. cfr-mediated multi-resistance

cfr-mediated multi-resistance was first detected on the multi-resistance plasmid pSCFS1 from a bovine *S. sciuri* isolate (Schwarz *et al*., 2000). Studies on the presence and distribution of the gene *cfr* identified this gene on plasmids or in the chromosomal DNA of *S. sciuri* from pigs and cattle from Germany (Kehrenberg and Schwarz, 2006), in *S. sciuri* from pigs in China (Wang *et al*., 2012; He *et al*., 2013), as well as in *S. sciuri* and *S. lentus* from chickens and in a *S. sciuri* from a duck from China (He *et al*., 2013). A recent study conducted in Belgium also identified single *cfr*-positive *S. sciuri* and *S. lentus* isolates from cattle (Vanderhaeghen *et al*., 2013). When located on a plasmid, the *cfr* gene is often part of multi-resistance plasmids that carry several other resistance genes (Kehrenberg *et al*., 2004; Wang *et al*., 2012; He *et al*., 2013).

3.6.7. Resistance to trimethoprim

Little information is currently available about *dfr* genes among members of the *S. sciuri* species group. One study identified the gene *dfrD* in a *S. sciuri* and a *S. fleurettii* as well as the gene *dfrG* in a *S. vitulinus* isolate, all from bovine milk (Frey *et al*., 2013). In another study, the gene *dfrA* was detected in a *S. vitulinus* and a *S. sciuri*, and the gene *dfrD* was found in two *S. vitulinus* and a *S. sciuri*, all from horses (Schnellmann *et al*., 2006).

3.6.8. Resistance to fusidic acid

Resistance to fusidic acid has been reported in *S. sciuri* isolates from a survey in the Indonesian population (Severin *et al*., 2010). Furthermore, *S. vitulinus* and *S. sciuri* isolates from the nasal cavity of various domestic animals were found to be fusidic acid-resistant (Bagcigil *et al*., 2007).

Although fusidic acid-resistant *S. sciuri* isolates (n=2) carrying the *fusB* resistance gene have been detected in horses (Aslantaş *et al*., 2012), this resistance is often found not to be associated with the known fusidic acid resistance genes *fusB*, and *fusC* (Frey *et al*. 2013).

Part II – Aims of the study

Methicillin resistance in staphylococci has become a worldwide concern in veterinary medicine. While MRSA is considered as an important pathogen in both human and veterinary medicine, other species such as *S. sciuri* are most often considered as harmless commensal bacteria though resistance genes have been detected previously in this species. However *S. sciuri* is very common in a broad range of habitats and is commonly found to be methicillin resistant. It may therefore represent a potential reservoir for genes encoding for instance antimicrobial resistance which might be transfert to more virulent staphylococci such as *S. aureus*. Epidemiology of MRSA and MRSS in healthy animals is still poorly understood. Therefore, the general aim of this study was to determine whether MRSS may be a reservoir for resistance and virulence genes for *S. aureus*.

The specific objectives of this research were:

- To estimate the prevalence and determine molecular epidemiology of MRSA isolatedfrom healthy carrier chickens **(Chapter 4)** as well as from healthy carrier bovines of different age group **(Chapter 5**).
- To explore antimicrobial resistance and virulence genes recovered from bovine MRSA **(Chapter 5**).
- To determine the molecular epidemiology of MRSS isolated from healthy carrier chickens and to assess the diversity of resistance and virulence gene encountered in these isolates **(Chapter 6**).
- To estimate the genetic diversity of MRSS isolated from healthy pigs, bovines and broiler chickens and to assess the role of MRSS as a potential resistance and virulence gene reservoir for other staphylococci **(Chapter 7)**

Part III – Experimental studies

Chapter 4 - Characterization of methicillin-resistant *Staphylococcus aureus* from healthy carrier chickens

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4.1. Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been recognized as an important pathogen in human medicine leading to hospital and community acquired infections. However, it is now also considered as a growing problem in veterinary medicine, though causing little to no infections. Although MRSA has already been detected in livestock including poultry, little is known about the epidemiology of MRSA in broiler and layer chickens. Therefore we investigated 372 poultry farms in Belgium. We also compared the isolation method recommended by the European Food Safety Authority (EFSA) using two enrichment steps with an isolation method using only one enrichment step. Isolated MRSA were characterized by means of antimicrobial resistance profiling, *spa* typing, multi locus sequence typing (MLST), and staphylococcal cassette cgromosome (SCC)*mec* typing. MRSA between herd prevalence was estimated at 3.3% for broiler herds using the double broth enrichment method, while using the single broth enrichment method it was estimated at 4.8% for broiler herds and 0.8% for layer herds. Five MRSA strains belonged to the livestock-associated (LA)-MRSA sequence type (ST)398 (four with *spa* type t011 and one with t899), and three to the hospital-acquired (HA)-MRSA ST239 *spa* type t037. The ST239 strains carried SCC*mec* type III while those belonging to ST398 carried SCC*mec* type IV or V. All isolates showed additional resistance to erythromycin and tetracycline apart from the expected resistance to cefoxitin and penicillin. All strains were susceptible to linezolid, mupirocin and vancomycin. In conclusion, a higher sensitivity for the isolation of LA-MRSA was obtained using only one enrichment step. While the typical LA-MRSA ST398 was present at low prevalence in poultry, also human associated strains have been found.

4.2. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are an important cause of hospital and community acquired infections worldwide (Stewart & Holt, 1963; von Eiff *et al*., 2001; Kluytmans-Vandenbergh *et al*., 2006). However, MRSA strains are not confined anymore to healthcare settings, and are nowadays a growing problem in veterinary medicine. In livestock, MRSA was first reported in a case of bovine mastitis (Devriese *et al*., 1972). It has been shown that this strain was a human associated strain. In animals, MRSA infections were mainly of human origin until 2005, when a high prevalence of a specific clone of MRSA was reported in pigs in the Netherlands. This clone was named later on "livestock associated MRSA" (LA-MRSA) and corresponded to the clonal complex (CC) 398 (Voss *et al*., 2005). Ever since, this clone has been found in many animal species, including poultry, all over the world (Persoons *et al*., 2009; Feβler *et al*., 2010; Graveland *et al*., 2010; Graveland *et al*., 2011; Crombé *et al*., 2012).

In poultry, a first report on MRSA came from South Korea in 2003 (Lee, 2003). The strains were both human and animal-associated. Other studies demonstrated MRSA in raw chicken meat (Kitai *et al*., 2005; Dohoo *et al*., 2009) and broilers at slaughterhouses (Mulders *et al*., 2010). LA-MRSA isolates in poultry have previously been found in The Netherlands (Leenders *et al*., 2007; Geenen *et al*., 2013) and Belgium (Nemati *et al*., 2008; Persoons *et al*., 2009; Vanderhaeghen *et al*., 2010a; Pletincks *et al*., 2011). However, these studies were rather limited and different isolation methods were used. Hence, a detailed understanding of the epidemiology of MRSA in poultry so far has been lacking. Therefore, a national survey on the prevalence of MRSA in both layers and broilers was conducted. Since isolation methods are of importance in prevalence estimations, we compared the method proposed by the European Food Safety Authority (EFSA) (Anon., 2009) with a less laborious modified method which will allow international comparisons.

4.3. Material and methods

4.3.1. Sample origin and isolation methods

A total of 372 farms, of which 92 were raising broilers and 280 were egg producing farms were sampled in 2011 all over Belgium. Following EFSA recommendations (Anon., 2012), this survey was conducted in conjunction with that of national *Salmonella* control programmes. Representative chickens subjected to official sampling in the course of *Salmonella* control programmes were then also sampled for MRSA. Sampling was performed by the Belgian Federal Agency for the Safety of the Food Chain (FASFC). In each farm, 20 chickens were nostrils swabbed. These 20 swabs were pooled per farm at the laboratory and two different isolation methods were used.

In the first isolation method proposed by the EFSA, pooled swabs were inoculated in Mueller-Hinton (MH) broth (Becton Dickinson, US) supplemented with NaCl (6.5%) at 37°C for 20 to 24h. One ml of this broth was added to Tryptic Soy Broth (TSB) supplemented with cefoxitin (3.5mg/l) and aztreonam (75mg/l) and incubated at 37°C overnight. Ten µl of this broth was then plated on MRSA-ID (bioMérieux, Marcy-l'Etoile, France) and incubated 48 hours at 37°C. At both 24 and 48 hours, plates were inspected and suspected colonies were purified on a Columbia Sheep Blood (CSB) agar plate (Bio Rad Laboratories, Nazareth Eke, Belgium). These plates were incubated overnight at 37°C (Anon., 2007). The alternative protocol was applied to 332 farms out of the 372 sampled farms, 81 raising broilers and 251 egg producing farms. Both methods were applied using the same MH broth in which swabs were pooled. This second isolation method differed from the above described protocol in that the second enrichment in antibiotic supplemented broth was omitted. For this reason, the first isolation method developed by EFSA will further be referenced as Double Broth Enrichment Method (DBEM) and the second one as Single Broth Enrichment Method (SBEM).

4.3.2. DNA extraction, MRSA identification and characterization.

DNA was extracted from all isolates as previously described (Vanderhaeghen *et al.*, 2010b). MRSA identification was performed using a triplex PCR, previously published by Maes *et al*. (2002). This PCR allows detecting the staphylococcal specific 16S rRNA gene, the *nuc* gene specific for *S. aureus*, and the presence of the *mecA* gene responsible for methicillin resistance.

All MRSA isolates were *spa* typed as previously described (Harmsen *et al*., 2003), using Ridom StaphType software (www.ridom.de/staphtype). CC398 PCR was performed on all MRSA following the protocol described by Stegger *et al*. (2011), which allows the rapid identification of the *S. aureus* ST398. MRSA isolates that were negative in the CC398 were subjected to multi locus sequence typing (MLST) (Enright *et al*., 2000). Sequences of internal fragments were then compared to the international database (http://saureus.mlst.net) to obtain the sequence type (ST). SCC*mec* typing of all MRSA was performed using the two multiplex PCRs (M-PCRs) to type the *mec*-complex and *ccr*-complex as described by Kondo *et al*. (2007). Appropriate control strains were used.

4.3.3. Determination of antimicrobial resistance.

Antimicrobial resistance was determined using the micro-broth dilution method (Sensititre, Trek Diagnostic Systems, Magellan Biosciences) following the manufacturer's instructions and using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. Data from the EUCAST minimal inhibitory concentration (MIC) distribution website was last accessed 30th November 2012 (http://www.eucast.org). The antibiotics tested were those included in the EUST custom panel plate for S*taphylococcus*. The MIC was defined as the lowest concentration by which no visible growth could be detected.

4.3.4. Statistical analysis

The Cohen's kappa coefficient was calculated and interpreted according to Landis and Koch, (1977) in order to compare the two isolation methods. This analysis was done on those 332 farms for which both isolation methods were available (Table 6). Since both SBEM and DBEM are under estimation, no gold standard was defined. All farms where at least one sample was found positive in at least one test were considered as true positive farms. The relative sensitivity, negative predictive value (NPV), likelihood ratio positive (LR+) and negative (LR-) of both methods were also calculated using the previously described formulae (Dohoo *et al*., 2009) and Win Episcope 2.0.

$$\text{Sensitivity} = \frac{\#True\ positives\ (TP)}{\#\ True\ positives\ (TP) + False\ negatives\ (FN)}$$

$$\text{NPV} = \frac{\#\ True\ negatives\ (TN)}{\#\ True\ negatives\ (TN) + False\ negatives\ (FN)}$$

Cohen’s Kappa, Pearson chi square and Fisher’s exact test were computed using IMB SPSS Statistics® Version 20.0.

Table 6. Comparison of methicillin-resistant *Staphylococcus aureus* isolates detected using Double Broth Enrichment Method (DBEM) or Single Broth Enrichment Method (SBEM).

		DBEM		Total number
		Number of positive results	Number of negative results	
SBEM	Number of positive results	1	5	6
	Number of negative results	2	324	326
Total number		3	329	332

4.4. Results

4.4.1. MRSA detection

Over 372 farms tested, all farms tested positive using the DBEM raised broiler chickens leading to a total between herd prevalence of 3.3% (95% CI [2.3 – 4.2]) for broiler farms (Table 8).

Conversely, using the SBEM (Table 7), two positive farms were raising layers and four were raising broilers. Between herd prevalence with SBEM (Table 8) can thus be split in broiler prevalence (4.8%, 95% CI [3.7 – 5.9]) and layer prevalence (0.8%, 95% CI [0.75 – 0.84]). Interestingly, only one farm was found positive by both methods. Two farms detected positive with the DBEM have not been detected with the other method, and five farms detected positive with the SBEM have not been detected with the other method (Table 7). At a total of eight farms, broilers and layers included were then found to be MRSA positive using either of both methods (Table 7).

Table 7. Total number and prevalence of methicillin-resistant *Staphylococcus aureus* positive farms using Double Broth Enrichment Method (DBEM) or Single Broth Enrichment Method (SBEM).

Isolation method	Number of positive farms	Farm type
DBEM	3	Broilers
SBEM	6	2 Layers and 4 broilers
DBEM and SBEM[a]	8	Layers and broilers

[a]DBEM and SBEM is the comparison of both methods used in parallel.n, total number of broiler or layer farms.

Table 8. Methicillin-resistant *Staphylococcus aureus* prevalence in different farms for the Double Broth Enrichment Method (DBEM) and the Single Broth Enrichment Method (SBEM).

Isolation method[a]	Broilers (n)	Layers (n)
DBEM	3.3 (n=92)	0.0 (n=280)
SBEM	4.8 (n=81)	0.8 (n=251)

[a]Prevalence in per cent is computed out of 92 broilers and 280 layers for DBEM, and computed out of 81 broilers and 251 layers for SBEM.

n, total number of broiler or layer farms.

Cohen's kappa coefficient (k) was 0.21, indicating a fair agreement between the two methods and Fisher's exact test shown no significant difference between both methods ($p>0.05$). Based on the assumption that all farms that tested positive in at least one test are true positive farms (n=8), the relative sensitivity of the DBEM and the SBEM method is 0.375 (95% CI [3.95 – 71.0] and 0.75 (95% CI [45.0 – 100.0]) respectively (Table 9). NPV of the DBEM and SBEM methods are 0.985 (95% CI [97.2 – 99.8]) and 0.994 (95% CI [98.5 – 100.0]) respectively. The LR+ for DBEM and SBEM methods was 25 and 125 respectively, whereas the LR- for DBEM and SBEM methods was 0.635 and 0.252 respectively. Negative predictive value (NPV) for both methods were not significantly different ($p>0.05$). However, there was a significant difference in MRSA prevalence between broiler and layer farms (Fischer exact $p<0.05$).

Table 9. Comparison of relative sensitivity, relative specificity, positive and negative predictive value for the isolation methods used in this study.

	DBEM (95% CI)	SBEM (95% CI)
Relative sensitivity	0.375 (3.9-71.0)	0.75 (45.0 – 100.0)
NPV	0.985 (97.2-99.8)	0.994 (98.5 – 100.0)

CI, confidence interval; DBEM, Double Broth Enrichment Method; NPV, Negative Predictive value; SBEM, Single Broth Enrichment Method.

4.4.2. MRSA characterization

Among the MRSA isolates recovered, three different *spa* types were detected; four strains belonged to t011, three to t037 and one to t899. The SBEM detected two t037, two t011, and one t899 while the DBEM detected one t037 and one t011. However, one t011 was detected by either of both methods. All t011 and the single t899 MRSA were isolated from broilers, but t037 strains were isolated from layers and broilers (Table 10). All t011 and t899 isolates were ST398, while the three MRSA type t037 strains belonged to ST239. These three strains carried SCC*mec* type III (3A), while the ST398 strains carried SCC*mec* IV (2B) or SCC*mec* V (5C2) cassettes.

Table 10. Genotyping of methicillin-resistant *Staphylococcus aureus.*

Strain	*spa* type	Sequence Type	*ccr* complex	*mec*	SCC*mec* type	Origin	Isolation method
M72	t037	ST239	A3/B3	A	III	Layer	SBEM
M86	t037	ST239	A3/B3	A	III	Layer	SBEM
M118b	t899	ST398	A2/B2	B	IV	Broiler	SBEM
M213	t011	ST398	A2/B2	B	IV	Broiler	SBEM + DBEM
M282	t037	ST239	A3/B3	B	III	Broiler	DBEM
M286	t011	ST398	C2	C	V	Broiler	DBEM
M363	t011	ST398	C2	C	V	Broiler	SBEM
M371	t011	ST398	A2/B2	B	IV	Broiler	SBEM

DBEM, Double Broth Enrichment Method; SBEM, Single Broth Enrichment Method; ST, sequence type.

All ST239 strains showed the same resistance profile and were resistant to cefoxitin, penicillin, erythromycin, tetracycline, chloramphenicol, kanamycin, rifampicin, sulfamethoxazole and streptomycin. None were resistant to ciprofloxacin, clindamycin,

fusidic acid, gentamicin, quinupristin/dalfopristin, tiamulin, and trimethoprim. All ST398 strains were resistant to cefoxitin, penicillin, erythromycin, tetracycline, clindamycin and trimethoprim. Four ST398 isolates showed resistance to gentamicin, kanamycin, ciprofloxacin and sulfamethoxazole. Two ST398 isolates were resistant to streptomycin, chloramphenicol, fusidic acid and tiamulin and one was resistant to rifampicin and quinupristin/dalfopristin (Table 11).

Table 11. MIC (mg/l) and antimicrobial resistance of all methicillin-resistant *Staphylococcus aureus* strains isolated in this study. Bold results indicate value above the EUCAST epidemiological cut offs (ECOFF).

Strains	CHL	CIP	CLI	ERY	FOX	FUS	GEN	KAN	LZD	MUP	PEN	RIF	SMX	STR	SYN	TET	TIA	TMP	VAN
M72	**64**	<=0.25	<=0.12	**>8**	**>16**	<=0.5	<=1	**>64**	2	<=0.5	**>2**	**>0.5**	**512**	**>32**	<=0.5	**>16**	<=0.5	<=2	<=1
M86	**64**	<=0.25	<=0.12	**>8**	**>16**	<=0.5	<=1	**>64**	<=1	1	**>2**	**>0.5**	**512**	**>32**	<=0.5	**>16**	<=0.5	<=2	<=1
M282	**64**	<=0.25	<=0.12	**>8**	**>16**	<=0.5	<=1	**>64**	<=1	<=0.5	**>2**	**>0.5**	**>512**	**>32**	<=0.5	**>16**	<=0.5	<=2	<=1
M118	<=4	**2**	**>4**	**4**	**16**	**1**	**8**	**64**	2	<=0.5	**>2**	0.03	<=64	8	1	**>16**	**>4**	**>32**	<=1
M213	**64**	0.5	**>4**	**>8**	**>16**	**2**	**>16**	**>64**	<=1	<=0.5	**>2**	**>0.5**	**256**	**>32**	<=0.5	**>16**	1	**>32**	<=1
M286	16	**2**	**>4**	**>8**	**16**	<=0.5	<=1	<=4	2	<=0.5	**>2**	<=0.016	<=64	8	1	**>16**	<=0.5	**>32**	<=1
M363	**32**	**2**	**>4**	**>8**	**16**	<=0.5	**>16**	**>64**	<=1	<=0.5	**>2**	<=0.016	**256**	**>32**	**4**	**>16**	**>4**	**>32**	<=1
M371	8	**>8**	**>4**	**>8**	**16**	<=0.5	**>16**	**64**	2	<=0.5	**>2**	<=0.016	<=64	8	1	**>16**	2	**>32**	<=1

CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, cefoxitin ; FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; LZD, linezolid; MIC, minimal inhibitory concentration ; MUP, mupirocin; PEN, penicillin; RIF, rifampicin; SMX, sulfamethoxazole; ST, sequence type; STR, streptomycin; SYN, quinupristin/dalfopristin; TET, tetracyclin; TIA, tiamulin; TMP, trimethoprim; VAN, vancomycin.

4.5. Discussion

In this study, we investigated 372 farms raising broilers or layers chickens in order to determine the MRSA prevalence. The between herd prevalence was low for both broiler and layer herds, ranging from 3.3% (95% CI [2.3 – 4.2]) to 4.8% (95% CI [3.7 – 5.9]) for broiler herds depending on the isolation method used. In our study we showed that MRSA prevalence was significantly higher in broiler farms compared to layer farms. This may explain the low overall prevalence, since 75% of sampled farms raised layers. Although the inclusion of a broth supplemented with aztreonam and cefoxitin has previously been considered as improving MRSA recovery (Böcher *et al*., 2010), the comparison between both isolation methods showed that using this broth may be too selective for MRSA detection in a low prevalence population. Indeed, only three farms were detected as positive with the DBEM method while SBEM method detected six farms. However, using the SBEM, more non *S. aureus* staphylococci were isolated (data not shown). Since these isolates were very similar to *S. aureus* on MRSA-ID this could lead to an extended lab work. Although the differences between the two methods are not statistically significant, the most sensitive isolation method is preferred, since it is important to detect as many positive farms as possible in a low prevalence environment to avoid the further spread to other farms. The same swabs were used for both isolation methods and the isolation steps were performed in parallel by the same technicians. Therefore, it is unlikely that the differences between methods were due to sampling or other accidental influences. An interesting finding in this study is the presence of the non ST398. Indeed, next to the classical MRSA ST398 *spa* type t899 and t011, three HA-MRSA ST239 *spa* type t037 with SCC*mec* type III were isolated. ST239 clones are disseminated worldwide and are among the oldest MRSA clones found in Europe (Monecke *et al*., 2011). They account for 90% of the HA-MRSA in Asia and have also been detected also in South America and are nowadays mainly circulating in Eastern Europe (Yamamoto *et*

al., 2012). Interestingly, ST239 shows geographic variations in terms of the *spa* type and the t037 found in this study is thought to be the ancestral ST239 *spa* type (Harris *et al*., 2010). This *spa* type has been recently reported in different countries as Malaysia and Russia (Neela *et al*., 2010; Yamamoto *et al*., 2012). Furthermore, while no MRSA were detected previously in layers (Persoons *et al*., 2009) MRSA ST239 was isolated both from broilers and layers farm.

All strains showed resistance to at least seven different antimicrobials and to a maximum of fourteen out of nineteen antimicrobials tested. As expected, all strains were resistant to penicillin and cefoxitin. All strains were also resistant to erythromycin and tetracycline. None was resistant to linezolid, mupirocin and vancomycin. In the recent study performed among poultry in Belgium by Persoons *et al*. (2009) all strains were susceptible to chloramphenicol, ciprofloxacin, quinupristin/dalfopistin and rifampicin. In contrast, in our study, four (80%) ST398 strains were resistant to ciprofloxacin, two (40%) were resistant to chloramphenicol and one to rifampicin and quinupristin/dalfopristin. Interestingly, the three ST239 shared the same resistance pattern, showing susceptibility to gentamicin, clindamycin and ciprofloxacin and resistance to chloramphenicol and rifampicin. These strains seem different from those isolated in Asia, where this clone is usually resistant to gentamicin, clindamycin and ciprofloxacin, and only in few cases resistant to rifampicin and susceptible to chloramphenicol (Kim *et al*., 2006).

Since MRSA ST239 *spa* type 037 is a hospital-acquired strain which is, at our knowledge, not reported in livestock, researchers and technicians that had worked in the laboratory during the surveillance were controlled in order to check their MRSA status. All were negative for MRSA. No information could be obtained about the MRSA status of the field workers or farmers. Furthermore, this *spa* type has not been recovered during the previous surveillance in hospital in Belgium (Stien Vandendriessche personal

communication). This is, to our knowledge, the first report of ST239 *spa* type t037 in Belgium.

4.6. Conclusion

MRSA prevalence in broiler farms was 3.3% with DBEM and 4.8% with SBEM which is significantly higher than that in layer farms. Nevertheless the overall between herd prevalence is low. Since broiler chickens have a higher prevalence than layers it is important to take this in account for proper prevalence determination. Prevalence should then be seen as a function of the sampling and isolation methods. The common LA-MRSA ST398 have been detected but we found for the first time HA-MRSA ST239 *spa* type t037 which is not common nor in livestock nor in the hospital according to the recent surveys conducted in Belgium. Yet the cause and origin of this clone in poultry is still unknown.

4.7. Acknowledgment

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Chapter 5 - Epidemiology and molecular characterization of methicillin-resistant *Staphylococcus aureus* nasal carriage isolates from bovines

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5.1. Abstract

Staphylococcus aureus is a common bacterium usually found on skin and mucous membranes of warm blooded animals. Resistance in *S. aureus* has been increasingly reported though depending on the clonal lineage. Indeed, while hospital acquired (HA)-methicillin resistant *S. aureus* (MRSA) are typically multi-resistant, community associated (CA)-MRSA is by large more susceptible to many antibiotics. Although *S. aureus* isolated from animals are often susceptible to most antibiotics, multi-resistant livestock associated (LA)-MRSA has been recovered from bovine mastitis.

In this study, we investigated the prevalence and types of MRSA present in the nose of healthy bovines of different age groups and rearing practices. Since no validated methods for MRSA isolation from nasal swabs were available, we compared two isolation methods. Molecular characterization was performed by means of *spa*-typing, multi locus sequence typing (MLST), staphylococcal cassette chromosome (SCC)*mec* typing and microarray analysis for the detection of antimicrobial resistance and virulence genes.

MRSA prevalence in bovines was estimated at 19.8%. There was a marked difference between rearing practices with 9.9%, 10.2% and 46.1% of the dairy, beef and veal calve farms respectively being MRSA positive. No significant difference was observed between both isolation methods tested. Most isolates were sequence type (ST)398 *spa* type t011 or closely related *spa* types. Few ST239 *spa* type t037 and t388 and ST8 *spa* type t121 were also found. SCC*mec* types carried by these strains were mainly type IV(2B), IV(2B&5) and type V. Type III and non-typeable SCC*mec* were recovered to a lesser extent. All isolates were multi-resistant to at least two antimicrobials in addition to the expected cefoxitin and penicillin resistance, with an average of resistance to 9.5 different antimicrobials. Isolates selected for microarray analysis carried a broad range of antimicrobial resistance and virulence genes.

MRSA were mainly present in veal farms, compared to the lower prevalence in dairy or beef farms. Multi-resistance in these strains was high. Though mainly clonal complex (CC)398 *spa* t011 was found, the genetic diversity was higher than what was found for pigs in Belgium. CC8 strains, a typically human lineage but also recently found also in association with bovines, has been retrieved here also.

5.2. Introduction

Staphylococcus aureus is a common facultative pathogenic bacterium that has long been recognized as a burden in both human and veterinary medicine. Indeed, *S. aureus* has been shown to be responsible of various infections such as clinical and subclinical bovine mastitis (Tenhagen *et al*., 2006; Vanderhaeghen *et al*., 2010b), wound infections in horses (Hartmann et *al*., 1997; Seguin *et al*., 1999; van Duijkeren *et al*., 2010), dogs (Gortel *et al*., 1999) and wild animals such as hedgehogs (Monecke *et al*., 2013a). Furthermore, *S. aureus* is well known to harbour resistance to antimicrobial agents which may lead to complications in the treatment of its infections (Lowy, 2003) and increase the cost of treatments (Huijps *et al*., 2008). One of these antimicrobial resistances is encoded by the *mecA* gene conferring resistance to almost all β-lactams including methicillin, oxacillin and cephalosporins. Though first considered not causing many infections (Devriese *et al*., 1972), methicillin resistant *S. aureus* (MRSA) have more recently been shown to be present in 10% of Belgian farms suffering from *S. aureus* bovine mastitis (Voss *et al*., 2005). In 2005, livestock associated (LA)-MRSA was first described in pigs and humans in close contact with pigs in the Netherlands (Armand- Lefevre *et al*., 2005) and in France (Baba *et al*., 2010). This particular clone belonging to the clonal complex (CC)398 was later encountered in many healthy animals such as pigs (Crombé *et al*., 2012), horses (Van den Eede *et al*., 2009), bovines (Graveland *et al*., 2010) and poultry (Persoons *et al*., 2009; Geenen *et al*., 2013; Nemeghaire

et al., 2014a). This clone complex is composed of different closely related *spa* types (Denis *et al*., 2009) and cannot be typed by pulsed field gel electrophoresis (PFGE) using SmaI digestion (Bens *et al*., 2006).

Although MRSA in bovines and in cases of bovine mastitis are well documented, information about the prevalence of *S. aureus* and MRSA in healthy bovines is lacking.

For international comparisons, a standardized isolation method is necessary. The European Food Safety Authority (EFSA) (Anon., 2009) has proposed a standardized protocol for the isolation of MRSA from dust samples obtained from pig farms. However, this protocol was estimated not to be very sensitive in a study in poultry in 2011 (Nemeghaire *et al*., 2013).

The aim of this study was to determine the prevalence and epidemiology of MRSA in bovines and assess the EFSA proposed isolation method with an alternative enrichment method in order to determine whether there were differences between the two methods in this population.

5.3. Methods

5.3.1. Sampling and isolation method

Four hundred and thirty-two farms were examined during the national survey on bovine MRSA in Belgium 2012. These farms were selected from the Sanitel database under stratified random sampling conditions. Of these, 141 were dairy farms, 187 farms reared beef cattle and 104 reared veal calves. Per farm, nose swabs were taken from 20 animals and pooled. Sampling was performed by the Belgian Federal Agency for the Safety of the Food Chain (FASFC).

The first method was the standard method proposed by ESFA (Anon., 2009), MRSA was isolated using Mueller-Hinton (MH) broth (Becton Dickinson, US) supplemented with NaCl (6.5%) and incubated at 37°C for 20 to 24h. One ml of this broth was added to Tryptic

Soy Broth (TSB) supplemented with cefoxitin (3.5mg/l) and aztreonam (75mg/l) and incubated overnight at 37°C. Ten µl of this broth was plated on MRSA selective plate, MRSA-ID (bioMérieux, Marcy-l'Etoile, France), and incubated 48 hours at 37°C. At both 24 and 48 hours, plates were inspected and suspected colonies were purified on Columbia agar plates with 5% sheep blood (CSB) (Bio Rad Laboratories, Nazareth Eke, Belgium) and incubated overnight at 37°C. Since this isolation method includes two enrichment steps, it is referred in this study as double broth enrichment method (DBEM).

The alternative method was applied to 106 farms and differed from the DBEM protocol by the omission of the second enrichment in antibiotic supplemented broth. For this reason, this second isolation method is referred as single broth enrichment method (SBEM).

5.3.2. DNA extraction, MRSA identification and characterization

DNA was extracted as previously described (Hartmann et *al*., 1997). MRSA identification and *mecA* gene detection was performed using a triplex PCR previously published (Maes *et al*., 2002).

A PCR allowing the detection of the clonal complex (CC) 398 was performed on all MRSA following a protocol previously described by Stegger *et al.* (2011). MRSA isolates that were negative in the CC398 PCR were subjected to multi-locus sequence typing (MLST) (Enright *et al*., 2000). Sequences of seven internal fragments were then compared to the international database (http://saureus.mlst.net) to obtain the sequence type. Strains were further characterised by *spa*–typing, as previously described (Harmsen *et al*., 2003). The resulting *spa type*s were assigned by using the Ridom StaphType software (www.ridom.de/staphtype). Clustering of *spa types* was performed using the algorithm Based Upon Repeat Pattern (BURP) available in the Ridom StaphType software. Staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by the means of two multiplex

PCRs (M-PCRs) designed for the detection of the *mec*-complex and *ccr*-complex (Kondo *et al*., 2007). Appropriate control strains were used.

5.3.3. Antimicrobial susceptibility testing

Antimicrobial resistance was determined using a micro broth dilution method (Sensititre, Trek Diagnostic Systems, Magellan Biosciences, Ohio, USA). The minimal inhibitory concentrations (MIC) of 19 antimicrobials (penicillin, cefoxitin, kanamycin, streptomycin, gentamicin, erythromycin, clindamycin, quinupristin/dalfopristin, linezolid, tiamulin, chloramphenicol, rifampicin, ciprofloxacin, fusidic acid, tetracycline, trimethoprim, sulfamethoxazole, vancomycin, and mupirocin) were determined as previously described (Denis *et al*., 2009). The MIC values were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-offs (ECOFF) for *S. aureus*. Data from the EUCAST MIC distribution website was last accessed November 6, 2013 (http://www.eucast.org).

5.3.4. DNA microarray-based typing and detection of resistance and virulence genes

Fourteen isolates were selected at random for detection of resistance and virulence genes by the mean of microarray analysis. Microarray analysis was performed on these strains using the Identibac *S. aureus* Genotyping DNA Microarray (Alere Technologies GmbH, Köln, Germany) according to the manufacturer's instructions. The DNA microarray covers 333 oligonucleotide probes, detecting resistance and virulence genes. A full list including primer and probe sequences is available online (http://alere-technologies.com).

5.3.5. Statistical analysis

The number of resistant strains was counted and resistance percentages were calculated. The Cohen's kappa coefficient was calculated in order to compare both isolation methods. Cohen's kappa coefficient was interpreted according to Landis and Koch (1977). This analysis includes the first 106 farms. Since both SBEM and DBEM are under estimations, no gold standard was defined. All farms that tested positive in at least one test were considered as true positive farms. Cohen's Kappa coefficient, relative sensitivity, and negative predictive value (NPV) of both methods were also calculated using a previously described formula (Dohoo *et al*., 2009) and Win Episcope 2.2 (Clive, United Kingdom). Pearson chi square and Fisher's exact test were computed using IMB SPSS Statistics® Version 20.0.

5.4. Results

5.4.1. Prevalence

Among the 81 farms tested positive using the official DBEM, 14 (9.9%, 95% CI [5.0% - 14.9%]) were dairy farms, 19 (10.2%, 95% CI [5.8% - 14.5%]) were farms holding beefs and 48 were (46.1%, 95% CI [36.6% - 55.7%]) farms rearing veal calves (Table 12).

Table 12. Methicillin-resistant *Staphylococcus aureus* in between herd prevalence in different farms.

Categories	n positive farms (N)	n negative farms (N)	Prevalence (%)	95% CI
Dairy cow	14 (N=141)	127 (N=141)	9.93	[4.99 - 14.9]
Veal calve	48 (N=104)	56 (N=104)	46.15	[36.6 - 55.7]
Beef cow	19 (N=187)	168 (N=187)	10.16	[5.83 - 14.5]
n total	81	351	18.75	[15.1 - 22.4]

CI, confidence interval; N, number of farm in the category; n number of positive or negative farm.

5.4.2. Comparison of isolation methods

Comparisons were performed on 106 samples. Using both isolation methods (Table 13), 34 (32.1%, 95% CI [23.2% - 41.1%]) farms out of 106 tested were found to be positive. Among these positive farms recovered, nine farms were detected positive with the SBEM but not with the DBEM and conversely, nine other farms were detected positive with the DBEM but not with the SBEM. Kappa agreement coefficient (k) was 0.61 which indicates a substantial agreement between both methods. There was no significant difference between the prevalence as established by both methods ($p > 0.05$). Negative predictive value were likewise identical (Table 14).

Table 13. Comparison of the number of methicillin-resistant *Staphylococcus aureus* isolates detected using Double Broth Enrichment Method (DBEM) or Single Broth Enrichment Method (SBEM).

		DBEM		Total
		Positive	Negative	
SBEM	Positive	25	9	34
	Negative	9	63	72
Total		34	72	106

Table 14. Comparison of the test evaluation of both isolation methods.

	DBEM (%)	SBEM (%)	95% CI Lower limit	95% CI Upper limit
Apparent prevalence	32.1	32.1	23.2	41.0
True prevalence	40.6	40.6	31.2	49.9
Relative sensitivity	79.1	79.1	66.9	91.2
Predictive value Negative	87.5	87.5	79.9	95.1

CI, Confidence interval; DBEM, Double broth enrichment method; SBEM, Single broth enrichment method.

5.4.3. MLST, spa- and SCCmec typing

Among 81 MRSA isolates recovered, seventy-eight (96.3%) were positive in the CC398 PCR. The three other isolates were ST8 and two ST239, as demonstrated by MLST.

All calf isolates were CC398. The ST8 was recovered from beef cattle and both ST239 isolates were isolated from dairy farms (Table 15).

Table 15. Total number of MRSA isolates corresponding to the different genotypes recovered and separated by farm types.

	MLST			*spa* types										SCC*mec*				
	8	239	398	t011	t037	t121	t388	t1451	t1456	t1985	t3423	t6228	NT	III (3A)	IV (2B)	IV (2B&5)	V (5C2)	NT
Dairy farms	0	2	12	8	1	0	1	0	1	0	0	2	1	1	4	0	6	3
Beef farms	1	0	18	16	0	1	0	0	1	1	0	0	0	1	12	0	3	3
Veal farms	0	0	48	40	0	0	0	3	1	3	1	0	0	0	30	11	7	0
Total	1	2	78	64	1	1	1	3	3	4	1	2	1	2	46	11	16	6

MLST, Multi locus sequence typing; NT, non-typeable

Ten different *spa* types were identified. Sixty-four (79.0%) were *spa* type t011. Other *spa* types recovered were t037 (n=1), t121 (n=1), t388 (n=1), t1451 (n=3), t1456 (n=3), t1985 (n=4), t3423 (n=1), t6228 (n=2) and a non-typeable *spa* type. Two clusters were distinguished using the BURP algorithm (figure 4).

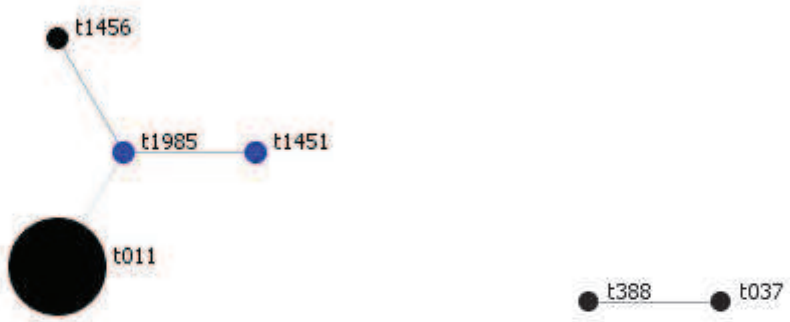

Figure 4. Clustering of *spa types* performed using Based Upon Repeat Pattern (BURP) algorithm.

The first cluster, including 92% of all isolates and 44% of all *spa* types, grouped the *spa* types t011, t1451, t1456 and t1985. The second cluster, which included 3% of all strains and 22% of all *spa* types, grouped the *spa* types t037 and t388. A singleton was also detected with the *spa*-type t121. The remaining *spa* types t3423 and t6228 could not be aligned by the software. All t011 and closely related *spa*-type isolates were associated to CC398. MRSA *spa* type t121 was associated to MLST type ST8, while t388 and t037 to ST239. The MRSA t011 and closely related strains were isolated from veal (n=47), beef (n=18) and dairy farms (n=9). The t3423 and t6228 MRSA were isolated from veal (n=1) and dairy farms (n=2). The t037, t388 and the non-typable *spa* type MRSA were recovered from dairy farms and the t121 MRSA was recovered in beef farm (Table 4).

Forty-four (54.3%) isolates carried SCC*mec* type IV(2B) and nine (11.1%) SCC*mec* type IV(2B&5). Sixteen (19.8%) isolates carried SCC*mec* type V(5C2) and two (2.5%) SCC*mec* type III(3A). Ten (12.3%) isolates were non-typeable using these M-PCRs. SCC*mec* type IV (2B and/or 2B&5) were found in isolates from veal (n=37), beef (n=12) and dairy

farms (n=4). SCC*mec* type V were also found in the three age groups with seven being found in isolates from veal, six from dairy and three from beef cattle. Type III cassette were found in from dairy (n=1) and beef cattle (n=1). The non-typeable SCC*mec* was detected in strain from veal calves (n=4), dairy (n=3) and beef (n=3) cattle. Additionally to the type IV(2B) (n=43), IV(2B&5) (n=9), V (n=16) and non-typeable (n=8) SCC*mec*, CC398 MRSA isolated also carried the type III (n=2) SCC*mec*. Both t121 and the non-typeable *spa* type carried SCC*mec* type IV(2b) and *spa* types t388 and t037 carried a non-typeable SCC*mec*.

5.4.4. Antimicrobial resistance

All strains were resistant to cefoxitin and penicillin as expected. More than 90% of the strains were resistant to tetracycline (96.3%) and trimethoprim (95.1%). A high prevalence of resistance was also observed to clindamycin (86.4%), erythromycin (86.4%), kanamycin (80.2%) and gentamicin (76.5%). More than half of the strains were also resistant to streptomycin (58.0%). Lower resistance levels were detected to fusidic acid (27.2%), sulfamethoxazole (25.9%), quinupristin/dalfopristin (23.5%), tiamulin (17.3%), ciprofloxacin (16.0%), chloramphenicol (12.3%), rifampicin (12.3%) and mupirocin (9.9%). No resistance was observed to linezolid and vancomycin (Table 16). All isolates were at least resistant to two more antimicrobials in addition to cefoxitin and penicillin. More than 50% of the strains were resistant to nine or more different antimicrobials. Two strains were resistant to 16 different antimicrobials, remaining susceptible only to ciprofloxacin, linezolid and vancomycin. The strains resistant to 15 (n=3) or 16 (n=2) antibiotics were all CC398 *spa* type t011. Two of these isolates carried a non-typable cassette and three carried SCC*mec* type IV (2B). These originated from veal (n=3) and beef cattle (n=2). The one strain resistant to 14 antibiotics was a CC398 *spa* type t6228 strain carrying SCC*mec* type V. The one strain resistant to only four antibiotics was a CC398 *spa* type t1456 strain carrying SCC*mec* type V

and originated from a farm holding beef cattle. Isolates that were resistant to five (n=1) and six (n=5) antimicrobials were CC398 *spa* type t011 carrying SCC*mec* type V (n=3) and IV (2B; n=1) or t1985 (n=2). These isolates were isolated from veal calves (n=3), dairy (n=1) and beef cattle (n=2). The ST8 isolate was resistant to seven different antimicrobials and both ST239 isolates were resistant to nine different antimicrobials. There were no significant differences in resistance prevalence between strains from veal calves, dairy and beef cattle.

Table 16. MIC distribution in methicillin-resistant *S. aureus* isolates from bovines.

Antimicrobials	% of isolates with MIC (mg/l) of																%R
	≤0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
CHL									4.9	46.9	35.8	2.5	8.6	1.2			12.3
CIP					18.5	27.2	9.9	2.5	2.5	11.1	28.4						16.0
CLI				12.3	1.2	1.2	1.2	0.0	0.0	84.0							86.4
ERY					3.7	7.4	2.5	0.0	2.5	2.5	81.5						86.4
FOX						0.0	0.0	0.0	0.0	3.7	18.5	77.8					100.0
FUS						72.8	12.3	2.5	9.9	2.5							27.2
GEN							22.2	1.2	3.7	7.4	21.0	44.4					76.5
KAN									16.0	3.7	2.5	2.5	9.9	65.4			80.2
LZD							23.5	75.3	1.2	0.0							0.0
MUP						86.4	3.7	3.7	0.0	0.0	0.0	0.0	0.0	0.0	6.2		9.9
PEN				0.0	0.0	0.0	1.2	8.6	90.1								100.0
RIF	86.4	1.2	0.0	1.2	0.0	1.2	9.9										12.3
SMX													70.4	3.7	11.1	14.8	25.9
STR									14.8	22.2	4.9	9.9	48.1				58.0
SYN						32.1	44.4	8.6	8.6	6.2							23.5
TET						2.5	1.2	0.0	0.0	0.0	1.2	95.1					96.3
TIA						75.3	7.4	0.0	0.0	17.3							17.3
TMP								4.9	3.7	1.2	0.0	1.2	88.9				95.1
VAN							87.7	12.3	0.0	0.0	0.0						0.0

CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, cefoxitin FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; LZD, linezolid; MIC, minimal inhibitory concentration; MUP, mupirocin; PEN, penicillin; R, resistance; RIF, rifampicin; SMX, sulfamethoxazole; STR, streptomycin; SYN, quinupristin/dalfopristin; TET, tetracyclin; TIA, tiamulin; TMP, trimethoprim; VAN,vancomycin.

Empty boxes indicate the concentration values that were not tested. Values in grey boxes indicate MIC higher than the concentration tested.

The bold lines indicate epidemiological cut-off values for *S. aureus*. MIC values were interpreted using the EUCAST clinical breakpoints /epidemiological cut-offs (http://www.eucast.org).

5.4.5. Microarray typing for resistance and virulence gene detection

Most genes were homogeneously distributed in all strains, including typical *S. aureus* species marker and regulatory genes (23S-rRNA, *gapA*, *katA*, *coA*, *nuc*, *spa*, *sbi*, *sarA*, *saeS, vraS*), the accessory gene regulator *agrI*, haemolysins (*hla*, *hld*), genes encoding leukocidins (*lukS*-F, *hlgA*, *lukX*, *lukY*-variant 1), proteases (*aur*, *sspA*, *sspB*, *sspP*), the biofilm production genes of the *icaACD* operon, adhesion factors (*bbp*, *cflA*, *cflB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sdrC*, *sdrD*, *vwb*) immune-evasion factors (*isaB*, *isdA, hysA1, hysA2*), a putative transport protein (*lmrP*), a site specific deoxyribonuclease subunit X (*hsdSx*), and staphylococcal superantigen-like proteins from the vSaα genomic islands [*setB1*, *setB2*, *setB3*, *setC*, *ssl1* (*set6*), *ssl2* (*set7*), *ssl4* (*set9*/*ssl4*), *ssl5* (*set3*/*ssl5*), *ssl7* (*set1*/*ssl7*) and *ssl10* (*set4*/*ssl10*)].

All strains were penicillin resistant and carried the *bla* operon (*blaZ, blaI,* and *blaR*) encoding for penicillin-ampicillin resistance. All isolates, except the tetracycline sensitive one, carried the tetracycline resistance gene *tetM.* Additionnaly to *tetM*, isolates harbouring SCC*mec* type V and a non-typeable isolate carried also the tetracycline resistance gene *tetK*. Six erythromycin resistant isolates out of 11 carried the *ermC* gene. Eight gentamicin resistance isolates out of the nine tested showed the aminoglycoside adenyl-/phosphotransferase encoding gene *aacA-aphD*. Eight kanamycin resistant isolates out of 11 carried the *aadD* aminoglycoside resistance gene and one additionally carried aminoglycoside phosphotransferase *aphA3*. One of the two chloramphenicol resistant isolate carried the *cat* (*pMC524*) gene encoding for chloramphenicol acetyltransferase. All isolates carried the putative transport protein *sdrM*. The metallothiol transferase (*fosB*) gene encoding fosfomycin resistance was detected in both non CC398 isolates. Furthermore, most isolates carried an intact beta-haemolysin gene (*hlb*), except the ST239 isolate which harboured the *hlb* gene

truncated after the probable insertion of the immune-evasion phage-borne genes *sak* (staphylokinase) and *scn* (staphylococcal complement inhibitor).

5.5. Discussion

In this study, we found 81 MRSA positive bovine farms in Belgium. As found in The Netherlands (Graveland *et al*., 2010) and a small former Belgian study (Vandendriessche *et al*., 2013), the prevalence in veal calve farms was much higher than in dairy farms or farms holding beef cattle. In contrast, the prevalence found at veal calve farms was lower than in these previous studies. In the Netherlands, MRSA prevalence in veal calve farms was estimated at 88% (Graveland *et al*., 2010) while the small scale Belgian study estimated a prevalence of 64% (Vandendriessche *et al*., 2013). The lower prevalence in our study may be explained by the differences in sampling. While in this study, swabs were pooled, in the other two studies, ten to 25 individual samples per farm were analyzed. Compared to other livestock animals, the estimated prevalence in bovines is much higher than that in poultry (0.8%) (Nemeghaire *et al*., 2013) but lower than that in pigs (68%) (Crombé *et al*., 2012).

The isolation method used throughout the study (the DBEM) was the method recommended by EFSA and the European reference laboratory. However, as shown for samples from poultry (Nemeghaire *et al*., 2013), representing a low prevalence population, the second enrichment method does not make any difference. Therefore we recommend for future European surveillances to use the SBEM on nasal swabs.

Most isolates were typical LA-MRSA CC398 *spa* type t011 or closely related *spa* types. Three other MLST types were recovered: ST8 *spa* type t121 and ST239 *spa* type t037 and t388. Those three types are usually identified among hospital-acquired (HA)-MRSA. However, while MRSA *spa* type t121 was uncommonly found in Belgian hospitals (Wildemauwe *et al*., 2010) it has been commonly recovered in hospitals in Europe and in the

United States (http://spa.ridom.de). This *spa* type has also been found in bulk tank milk in the United States (Haran *et al*., 2012). MRSA ST239 *spa type* t388 and t037 are widespread HA-MRSA found in Europe, Asia and America (Campanile *et al*., 2010). These two ST239 MRSA isolates were found in different neighbouring provinces of Belgium being East Flanders and Flemish Brabant. A MRSA ST239 t037 was also isolated from poultry in 2011 (Nemeghaire *et al*., 2013). The recovery of these HA-MRSA from livestock indicates that one should remain vigilant to the evolution of MRSA in animals. Though not investigated in his study, these strains in general carry a multitude of virulence genes on mobile genetic elements. Transfer of these virulence genes to LA-MRSA CC398 would have a huge impact on the importance of this clone for human health and its epidemiology in animals.

The diversity of *spa* types seen in this study in bovines was larger than what has been found previously in pigs in Belgium, where only *spa* type t011 and t034 were found (Crombé *et al*., 2012; http://www.efsa.europa.eu/en/efsajournal/pub/1597.htm]. In bovines, at least seven different *spa* types were recovered among the MRSA CC398 isolates. It has been concluded previously that the length of the *spa* gene sequence may depend on the fact that isolates are methicillin resistance or not, or on the source of *S. aureus* isolation (Shakeri *et al*., 2010). Since our isolates were all methicillin resistant and *spa*-types were found to be closely related, the hypothesis of a possible host adaptation is supported. Also the diversity of the SCC*mec* types in strains from cattle seems to be larger than what is found in pigs, however the two predominant types are the same, SCC*mec* type IV and SCC*mec* type V. Surprisingly, two isolates carried SCC*mec* type III. This type is typically associated with HA-MRSA (Moroney *et al*., 2007) and has also been found extensively in *Staphylococcus* spp. other than *S. aureus* from animals. SCC*mec* type III has been described before in ST398, but these were in fact variant SCC*mec* type V (van Loo *et al*., 2007; Jansen *et al*., 2009). Next to this, six isolates carried a non-typeable SCC*mec* cassette. Further studies are needed to be able to

estimate the plasticity of the SCC*mec*, since this may be of importance to the epidemiology of MRSA in livestock and humans.

The level of multi-resistance is extremely high since it accounts for an average of 9.5 different antimicrobials. Most isolates were resistant to tetracycline and trimethoprim additionally to the expected resistance to cefoxitin and penicillin. In this study two CC398 isolates were found to be susceptible to tetracycline while tetracycline susceptible strains are only very rarely found in CC398 MRSA (Kadlec and Schwarz, 2009a). The prevalence of erythromycin, clindamycin, kanamycin and gentamicin resistance in this collection is extremely high compared to what has been found in strains from other origins in Belgium (%) (Crombé *et al*., 2012; Vandendriessche *et al*., 2013). The isolate with the lowest level of multi-resistance was resistant to two additional antimicrobials. Two isolates were resistant to sixteen antimicrobials out of nineteen tested excluding ciprofloxacin, linezolid and vancomycin, three antimicrobials that are used as a last resort in the treatment of MRSA infections in humans.

Only one isolate carried immune evasion cluster (IEC) genes *sak*, *scn* and *sea* encoding staphylokinase, staphylococcal complement inhibitor and enterotoxine A, repectively. This IEC is carried on a bacteriophage of the φ3 family which is commonly found in human isolates but few in isolates from animals (Haenni *et al*., 2011) or humans in contact with pigs (Sung and Lindsay, 2007; McCarthy *et al*., 2012) and is known to play an important role in human colonization. Since these genes were found only on the ST239 isolate and not on the most typical ST398 LA-MRSA, this might indicate a human to animal transmission of non CC398 isolates. Most resistance and virulence gene detected were homogeneously distributed amongst isolates except for the macrolide/lincosamide resistance encoding gene *erm(C)* which was found in more than half of the erythromycin resistant isolates and the fosfomycin resistance gene *fosB* which was detected in two non CC398 isolates. However, the

presence of *fosB* cannot be compared to the phenotypic resistance since this fosfomycin was not included in the international Sensititre ® plate format. Additionally to resistance genes, virulence factors such as leukocidins, proteases, staphylococcal superantigen like proteins, haemolysins genes, genes involved in adhesion and immune-evasion were also found in all isolates tested by micro-array. Our results are similar to those of a previous micro-array based study performed in Germany (Monecke *et al*., 2007) on *S. aureus* isolates from cattle in which leukicidins, haemolysins and enterotoxin genes were detected in most isolates. According to this study, staphylokinase (*sak*) was also absent in most of our isolates except for the ST239 isolate. However, while in the German study toxic shock syndrome toxins, were demonstrated, the *tst*-1 gene was not detected in our isolates. Additionally, genes encoding adhesion factors including the bone sialoprotein-binding protein (*bbp*), the cell wall associated fibronectin-binding protein (*ebh*), the fibrinogen binding protein (*fib*), the fibronectin-binding protein (*fnbB*) and the major histocompatibility complex class II analog protein (*map*) were detected in all isolates. These genes were also found in MRSA isolates from Sahiwal cattle with mastitis in India (Kumar *et al*., 2011). Our results show that, although our isolates came from apparently healthy carrier animals, MRSA in bovines may carry a broad range of different resistance genes and virulence factor that might play an important role in the pathogenicity of the bacteria.

5.6. Conclusion

In conclusion, MRSA were found in bovines in different rearing practices. Estimated prevalence was, however, lower in nasal isolates from dairy and beef cows than from veal calves. No significant difference was observed between both isolation methods tested. The diversity of strains was larger than what was seen in pigs. Indeed, more different *spa*-types were recovered in bovine's isolates than in pigs. Additionally, the diversity in SCC*mec*

cassettes in CC398 was shown not to be limited to the types IV and V but included also type III and a non-typeable cassette. A high level of multi-resistance was found and a broad range of antimicrobial resistance and virulence genes was detected though animals sampled were apparently healthy.

5.7. Acknowledgments

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Chapter 6 - Molecular epidemiology of methicillin-resistant *Staphylococcus sciuri* in healthy chickens

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6.1. Abstract

Staphylococcus sciuri is commonly found on the skin of animals and humans as well as in the environment. However, little is known on its prevalence, resistance and epidemiology. Therefore, we investigated the prevalence of methicillin resistant *S. sciuri* (MRSS) strains in poultry, as they may represent a reservoir of resistance genes for other strains. In 2011, 281 poultry farms were sampled by taking nasal swabs of 20 animals. The swabs were pooled and MRSS were selectively isolated. Genus and methicillin resistance were determined by PCR and species identification was performed using transfer RNA-intergenic spacer analysis. MRSS were further characterised by Staphylococcal cassette chromosomr (SCC)*mec* typing, pulsed field gel electrophoresis (PFGE), microarray and susceptibility testing.

Eighty-seven MRSS were isolated resulting in an estimated between herd prevalence of 31.0%. The prevalence in broiler herds did not significantly differ from that in layer herds. Most isolates harboured a non-typeable SCC*mec* and a little less than 40% carried SCC*mec* type III. Isolates from broiler herds carried mostly the SCC*mec* type III, while isolates from layer herds carried mostly the non-typeable SCC*mec* cassette. The 87 isolates generated 47 different SmaI-PFGE profiles that grouped in two main clusters corresponding to the two farm types. All isolates were resistant to fusidic acid, tiamulin and gentamicin and were sensitive to rifampicin and vancomycin. Isolates selected for microarray analysis carried a broad range of antimicrobial resistance and virulence genes.

This study showed that MRSS is carried by healthy chickens at the same level in both broilers and layers. They represent a large reservoir for resistance and virulence genes. Strains from layers and broilers represent different clusters.

6.2. Introduction

Staphylococcus sciuri is thought to be the ancestral staphylococcal species. It is commonly found on skin and mucous membrane of warm blooded animals (Adegoke, 1986; Hauschild and Schwarz, 2003; Huber *et al.,* 2011) as well as in the environment (Pioch *et al*., 1988) and on humans (Shittu *et al.,* 2004). Previously considered as a non-pathogenic commensal bacterium, it has also been associated with animal diseases such as mastitis in dairy cattle (Rahman *et al*., 2005), dermatitis in dogs (Hauschild & Wójcik, 2007) and exudative epidermitis in piglets (Chen *et al*., 2007). *S. sciuri* is also known to be responsible for various infections in humans such as endocarditis (Hedin and Widerström, 1998), wound infections (Kolawole and Shittu, 1997), peritonitis (Wallet *et al.*, 2000) septic shock (Horii *et al.,* 2001) and urinary tract infections (Stepanović *et al.,* 2003).

It has been demonstrated that *S. sciuri* carries a close homologue of the *Staphylococcus aureus* methicillin-resistance gene *mecA* (Wu *et al.,* 1996), which does not confer resistance to β-lactam antibiotics (Couto *et al.,* 1996). Nevertheless *S. sciuri* may carry an additional staphylococcal cassette chromosome *mec* (SCC*mec*) harbouring the *mecA* gene (Archer and Niemeyer, 1994) and may thus represent a reservoir for methicillin resistance genes for other staphylococci such as *S. aureus.* However, little is known on its epidemiology. The aim of this study was to determine the prevalence of MRSS in healthy chickens and to assess its genetic diversity.

6.3. Material and methods

6.3.1. Sampling and isolation methods

In 2011, 281 poultry farms were sampled in different parts of Belgium. Following EFSA recommendations (Anon., 2012), this survey was conducted in conjunction with that of national *Salmonella* control programmes. Representative chickens subjected to official sampling in the course of *Salmonella* control programmes were then also sampled for MRSA. Sampling was performed by the Belgian Federal Agency for the Safety of the Food Chain Two-hundred and five were egg producing and 76 were broiler farms. Twenty chickens per farm were sampled in nostrils. These 20 samples were pooled per farm and incubated in Mueller-Hinton (MH) broth supplemented with NaCl (6.5%) at 37°C for 20-24h. Ten µl of this broth was plated on a methicillin resistant *Staphylococcus aureus* (MRSA) selective plate, MRSA-ID (bioMérieux, Marcy-l'Etoile, France), and incubated 48 hours at 37°C. At both 24 and 48 hours, plates were inspected and suspected colonies were purified on Columbia agar plates with 5% sheep blood (CSB) (Bio Rad Laboratories, Nazareth Eke, Belgium) and incubated overnight at 37°C (Anon, 2007).

6.3.2. Identification, mecA detection and SCCmec typing

DNA was extracted as previously described (Vanderhaeghen *et al*., 2010b). The detection of the *mecA* gene was performed using a triplex PCR previously published by Maes *et al*. (2002). Identification at the species level was performed by tDNA intergenic spacer analysis (Supré *et al*., 2009). For this, a PCR using degenerate primers directed outwards of the tRNA genes was executed using HiFi Supermix (Invitrogen, Ghent, Belgium), fluorescently labelled (T3B fluo) and unlabelled primer (T3B) and T5A primer. The PCR products were then sized using capillary electrophoresis on a CEQ8000 instrument (Beckman Coulter, Suarlée, Belgium). Finally, results were analysed using the BaseHopper software

(Ghent University). All isolates confirmed to be MRSS were typed in order to determine the SCC*mec* type (Kondo *et al*., 2007). Appropriate control strains were included for the SCC*mec* typing.

6.3.3. Macrorestriction-PFGE analysis

Whole genome DNA of isolates was prepared, digested by restriction enzyme SmaI and a Pulse field gel electrophoreses (PFGE) was performed using a CHEF Mapper system (Bio-Rad Laboratories, United Kingdom). Plugs were prepared according to the protocol of Argudín *et al.* (2010) with modifications. A two hours lysis step at 37°C with a solution composed of 6mM Tris/HCl, 1M NaCl, 100mM EDTA, 5.0 g/l N-laurylsarcosine (Sigma-Aldrich, Diegem, Belgium) and 1.0 g/l lysozyme (Sigma-Aldrich, Diegem, Belgium) was added prior to the lysis step (using 0,5M EDTA, 1% laurilsarcosine, and 1mg/ml proteinase K). Plugs were then subjected to restriction with SmaI (Fermentas GmbH, Belgium) following the manufacturer's instructions. The electrophoresis conditions were 6 V/cm in 0.5x TBE (45mM Tris, 45mM boric acid, 1mM EDTA [pH 8]) at 11.3°C and runs lasted 23 h with switch times from 5s to 35s. PFGE profiles were compared using BioNumerics software (Version 6.6, Applied Maths, Belgium). A dendrogram was derived from Dice similarity indices based on the unweighted pair group method with arithmetic averages (UPGMA). *S. aureus* NCTC 8325 (National Collection of Type Cultures, United Kingdom) was included as control strain for PFGE analysis.

6.3.4. Antimicrobial susceptibility testing

Antimicrobial resistance was determined using broth microdilution (Sensititre, Trek Diagnostic Systems, Magellan Biosciences, Ohio, USA) following the manufacturer's instructions. Susceptibility was tested for 19 antibiotics [penicillin (PEN), cefoxitin (FOX),

kanamycin (KAN), streptomycin (STR), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), quinupristin/dalfopristin (SYN), linezolid (LZD), tiamulin (TIA), chloramphenicol (CHL), rifampicin (RIF), ciprofloxacin (CIP), fusidic acid (FUS), tetracycline (TET), trimethoprim (TMP), sulfamethoxazole (SMX), vancomycin (VAN) and mupirocin (MUP)]. Concentrations tested are shown in table 17. The minimal inhibitory concentration (MIC) was defined as the lowest concentration by which no visible growth could be detected. The MIC values were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-offs (ECOFF) for coagulase-negative staphylococci (CoNS). In the case there were no ECOFF available, ECOFF for *S. aureus* were used. Data from the EUCAST MIC distribution website was last accessed October 2, 2013 (http://www.eucast.org). When no ECOFF values for CoNS or *S. aureus* were available, wild type and non-wild type determination was judged on the distribution of the strains over the MIC's as previously described (Butaye *et al.,* 2003). Additionally, in the following sections, the term resistance will refer to the microbiological resistance determined by the non-wild type distributions of the MIC's. Indeed, since results are observed from an epidemiological point of view, ECOFF values were preferred.

6.3.5. DNA microarray-based typing and detection of resistance and virulence genes

Thirty isolates were selected based on the antimicrobial resistance phenotypes and PFGE profiles. As such, isolates that were separated in different clusters at 80% similarity index and that showed different antimicrobial resistance profiles were selected. Microarray analysis was performed on these strains using the Identibac *S. aureus* Genotyping DNA Microarray (Alere Technologies GmbH, Köln, Germany) according to the manufacturer's instructions. The DNA microarray covers 333 oligonucleotide probes, detecting resistance and

virulence genes. A full list including primer and probe sequences is available online (http://identibac.com/en/home.html).

6.4. Results

6.4.1. MRSS prevalence among poultry farms

Eighty-seven MRSS were isolated among the 281 farms. Twenty-six were isolated from broiler farms and 61 from layer farms. Using this methodology, MRSS between herd prevalence in chicken farms was estimated at 31.0 % (95% CI [25.5-36.3]). Prevalence for broiler farms was 34.2% (95% CI [23.5-44.9]) and 29.8% (95% CI [23.5-36.0]) for layer farms.

6.4.2. SCCmec and macrorestriction-PFGE analysis

Thirty-four (39.1%) MRSS carried SCC*mec* type III (3A) and 53 (60.9%) showed only the *mec*A gene but no *ccr* complex was detected, and were thus considered non-typeable. Forty-seven (77.0%) isolates from layer farms carried a non-typeable SCC*mec* cassette and fourteen (23.0%) carried the SCC*mec* type III. On the contrary, isolates from broiler farms harboured mostly SCC*mec* type III (76.9%) and only 6 (23.0%) carried a non-typeable cassette. The 87 MRSS isolates generated 47 SmaI-PFGE profiles. On the basis of the Dice's similarity coefficient, a dendrogram with the 47 PFGE patterns was constructed (figure 5). At a similarity index of 40% the profiles were grouped in two different clusters. The first cluster grouped 32 different profiles and was composed of 58 isolates. Among these, 53 (91.4%) were layer farm isolates and five (8.6%) isolates were from broiler farms. In this cluster, 46 (79.3%) isolates carried a non typable SCC*mec* and twelve (20.7%) the SCC*mec* type III. The second cluster grouped 15 different profiles and was composed of 29 isolates. Twenty-two (75.9%) were isolates from broiler farms and seven (24.1%) from layer farms. Twenty-two isolates (75.9%) carried the SCC*mec* type III and seven (24.1%) a non-typeable SCC*mec*. The first cluster was divided in three sub-clusters; 1a, 1b and 1c. The sub-cluster 1a grouped 36

(62.0%) isolates and was composed of mostly layer farm isolates and only one isolate from a broiler farm. The sub-cluster 1b was composed of eleven (19.0%) isolates that originated only from layer farms. The last sub-cluster, 1c, also grouped eleven (19.0%) isolates and was composed of eight (72.7%) layer farm isolates for three isolates (27.3%) from broiler farms.

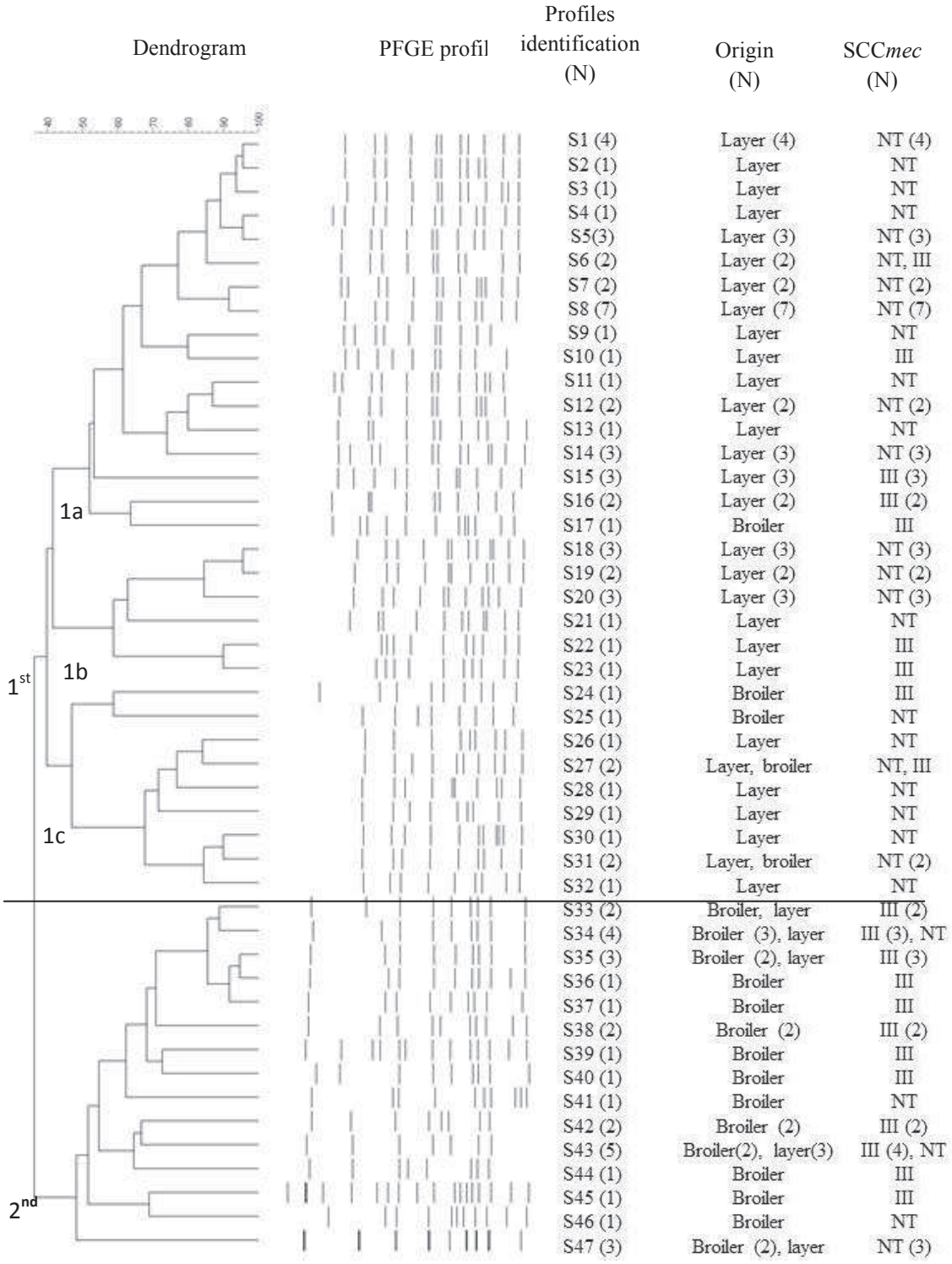

Figure 5. Dendrogram derived from Dice similarity indices based on the unweighted pair group method with arithmetic averages (UPGMA). The horizontal line shows the separation of the two major clusters. 1st, first cluster; 2nd, second cluster. N, number of isolates when more than one; NT, non-typeable; S, SmaI-PFGE profile

6.4.3. Antimicrobial resistance

Obviously, all 87 isolates were resistant to cefoxitin and penicillin. They were also all resistant to fusidic acid, tiamulin and gentamicin. Most strains showed also resistance to clindamycin (98.9%, 95% CI [98.6 – 99.1]), quinupristin/dalfopristin (97.7%, 95% CI [97.4 – 98.0]) and trimethoprim (86.2%, 95% CI [85.4 – 87.0]). Less than half of the strains showed resistance to tetracycline (40.2%, 95% CI [39.1 – 41.3]), mupirocin (27.6%, 95% CI [26.6 – 28.6]) and erythromycin (20.7%, 95% CI [19.6 – 21.6]). Few isolates were resistant to chloramphenicol (6.9%, 95% CI [6.3 – 7.5]), ciprofloxacin (5.8%, 95% CI [5.2 – 6.3]), streptomycin (5.7%, 95% CI [5.2 – 6.3%]), linezolid (3.5%, 95% CI [3.0 –3.9]), sulfamethoxazole (1.2%, 95% CI [0.9 – 1.3]) and kanamycin (1.1%, 95% CI [0.9 – 1.3]). None were resistant to rifampicin and vancomycin (Table 17). All isolates were resistant to at least seven antimicrobials and to a mean of 8.9 antimicrobials.

Table 17. MIC distribution of methicillin-resistant *S. sciuri* from chickens

AM tested	% of isolates with MIC (mg/l) of																R%
	<=0.0016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
CHL									64.40	28.70	0.00	2.30	4.60				6.90
CIP					4.60	56.32	33.33	2.30	3.45	0.00							5.75
CLI				0.00	1.15	31.04	42.53	5.74	0.00	19.54							98.85
ERY					28.74	50.57	0.00	1.15	0.00	0.00	19.54						20.69
FOX						0.00	0.00	0.00	0.00	1.10	13.80	85.10					100.0
FUS						0.00	0.00	3.40	87.40	9.20							100.0
GEN							98.90	1.10	0.00	0.00	0.00						100.0
KAN									96.60	2.30	1.10	0.00	0.00				1.10
LZD							44.83	51.72	2.30	1.15							3.45
MUP						70.12	2.30	10.34	0.00	0.00	0.00	0.00	0.00	0.00	17.24		27.58
PEN				0.00	0.00	0.00	1.15	14.94	83.91								100.0
RIF	100,00	0.00	0.00	0.00	0.00	0.00											0.00
SMX													97.70	1.15	0.00	1.15	1.15
STR									92.00	2.30	0.00	1.10	4.60				5.70
SYN						0.00	2.30	86.21	8.05	3.44							97.70
TET						58.62	1.15	1.15	0.00	0.00	0.00	39.08					40.23
TIA						0.00	0.00	0.00	0.00	100.0							100.0
TMP								13.80	67.81	1.15	0.00	0.00	17.24				86.20
VAN							97.70	2.30	0.00	0.00	0.00						0.00

AM, antimicrobial; MIC, minimal inhibitory concentration; R, microbiological resistance; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, cefoxitin FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; LZD, linezolid; MIC, minimal inhibitory concentration ; MUP, mupirocin; PEN, penicillin; RIF, rifampicin; SMX, sulfamethoxazole; STR, streptomycin; SYN, quinupristin/dalfopristin; TET, tetracyclin; TIA, tiamulin; TMP, trimethoprim; VAN,vancomycin.

Empty boxes indicate the concentration values that were not tested. Values in grey boxes indicate MIC higher than the concentration tested.

The bold lines indicate epidemiological cut-off values for *S. aureus*. The pointed lines indicate epidemiological cut-off values for coagulase-negative staphylococci while different of the *S. aureus*. MIC values were interpreted using the EUCAST clinical breakpoints /epidemiological cut-offs.

6.4.4. Microarray typing for resistance and virulence gene detection

Thirty isolates with different PFGE profiles at 80% similarity and different antimicrobial resistance profiles were selected for microarray analysis. Additionally to a selection of resistance and virulence genes, the array included also SCC*mec* typing analysis. The thirty isolates carried *mecA*, together with other SCC*mec* genes such as *mecR*, Δ*mecR*, *mecI* and *ccrA*-3. One isolate was also positive for the glycerophosphoryl diester phosphodiesterase, associated with *mecA* gene, *ugpQ*. Those isolates carrying SCC*mec* type III were also positive for *ccrB*-1 and *ccrB*-3. The penicillin-ampicillin resistance operon *bla* (*blaZ, blaI,* and *blaR*) was found in only four isolates and in one only *blaI* was detected. Among the eight erythromycin resistant isolates, six carried *erm*(B), one carried *erm*(C) and one had an ambiguous result for *erm*(C). Resistance to clindamycin and quinupristin/dalfopristin was also high, though few isolates carried typical *S. aureus* lincosamide and streptogramin resistance genes *vga*(A) and *vgb*(A). Furthermore, although all isolates were resistant to gentamicin according to the breakpoint, only five isolates showed the aminoglycoside adenyl-/phosphotransferase encoding gene *aacA-aphD*. Five kanamycin resistant isolates were positive for the *aadD* aminoglycoside resistance gene. Among the fourteen tetracycline resistant strains, two carried *tet*(K) and ten *tet*(M). In two isolates, no resistance gene was detected. Only one isolate of the three chloramphenicol resistant isolates carried the chloramphenicol acetyltransferase (*cat*) gene. Eleven isolates carried the *qacC* efflux pump gene encoding resistance against quaternary ammonium disinfectants. Finally, the only linezolid resistant isolate carried the *cfr* linezolid resistance encoding gene. This isolate was also resistant to tiamulin, quinupristin/dalfopristin, chloramphenicol and clindamycin.

Most virulence genes were heterogeneously distributed among isolates. The enterotoxin encoding gene *sed*, arginine catabolic mobile element (ACME) locus *arcD-SCC*,

capsule encoding gene *capK1* and the biofilm formation associated gene *bap* were found in a maximum of four (13%) isolates. Less than 15 (50%) isolates harboured the putative transporter encoding gene *lmrP*, the site specific deoxyribonuclease *hdsS2* and *hdsSx* and the immune-evasion factor *isdA*. Other virulence genes such as the leukocidin encoding gene *lukS*, adhesion factors *fnbA*, *bbp* or *ebpS*, the immune-evasion factor *isaB*, the staphylococcal superantigen-like proteins *ssl10* (*ssl10/set4*), and the protease *sspP* were carried by more than 22 (70%) of isolates.

6.5. Discussion

S. sciuri is a widespread bacterium commonly found in a wide range of habitats. First considered as a commensal bacterium associated with healthy or diseased farm and wild animals (Kloos, 1980), it is has also been recovered from hospital environment (Dakić *et al.,* 2005) and hospitalized patients (Jain *et al*., 2004). In the present study we investigated the prevalence of MRSS in healthy broilers and layers in order to verify whether they may be a reservoir of virulence and resistance genes and to determine the clonality of the methicillin resistant strains.

We found that 31.3% of the poultry farms were positive for MRSS using selective isolation and no significant differences were found between broiler and layer farms. Few studies dealt with the prevalence of methicillin resistant coagulase negative staphylococci (MRCoNS) and even less focussed on MRSS. Similar studies, also using selective isolation, have been performed. However, these studies focussed on MRCoNS rather than MRSS. Kawano *et al*. (1996) found a MRCoNS prevalence of 25.7% in chickens, Busscher *et al*. (2006) of 22.5% in horses, Vanderhaeghen *et al*. (2012b) of 30.3% in veal calves and 24.8% in beef cows and at 13% for both mares (Yasuda *et al*., 2002) and dogs (Bagcigil *et al*., 2007). In all of these studies, *S. sciuri* was often the most abundant species encountered.

The poultry farms analysed in this study were previously investigated for the presence of methicillin resistant *S. aureus* (MRSA). In this study, the MRSS prevalence was substantially higher than that of MRSA (1.8%) prevalence estimated in the same population (Nemeghaire *et al.*, 2013). This may indicate that either *S. aureus* is less prevalent in poultry or that *S. sciuri* acquires SCC*mec* more easily. Further studies should elucidate this.

The SCC*mec* cassette analysis revealed that SCC*mec* type III was common in the collection, and was carried by approximately 40% of isolates. Damborg *et al.* (2009) previously reported a high prevalence (78%) of this type in MRCoNS from animals. In our study most isolates (60%) carried a non-typeable SCC*mec* type. This non-typeable SCC*mec* cassette possessed a type A *mec* element and a *ccr* element could not be detected by the PCR described by Kondo *et al*. (2007). In contrast, the *ccrA* element was detected in 16 of the non-typeable isolates tested by microarray. This indicates that those isolates may carry a *ccrA* element with an aberrant sequence. Non-typeable cassettes have previously been found in MRCoNS isolates from pigs (18.1%) among which *S. sciuri* (n=12) were also recovered (Vanderhaeghen *et al*., 2012b). The high prevalence of non-typeable SCC*mec* cassettes in *S. sciuri* confirms the potential divergence of *ccr* and *mec* complexes as previously suggested (Urushibara *et al.*, 2011).

Since PFGE with SmaI restriction has been proved to have a high discriminative power in *Staphylococcus aureus* (Murchan *et al*., 2003) and has previously been applied to *S. sciuri* isolates from hospital environment (Dakić *et al.,* 2005), we estimated this method useful to assess the epidemiological relatedness between different *S. sciuri* strains. Indeed, in this study we showed a high diversity of MRSS in chicken farms since 47 different profiles were identified among the 87 isolates encountered in the different farms sampled. Furthermore, two large clusters were found. One cluster was composed of mainly layer hen isolates while the other only of isolates from boilers. This indicates that those isolates might

belong to different ecological MRSS populations. This may be explained by the fact that broilers and layer chickens are two different populations reared separately in order to select characteristics that are important for meat or egg production. Also the SCC*mec* distribution followed the PFGE clusters, giving an indication of the limited spread of SCC*mec* types between ecosystems.

All strains were resistant to fusidic acid, tiamulin and gentamicin. However, these resistances could not be attributed to the typical *S. aureus* acquired resistance genes tested. Indeed, all isolates were negative for the detection of fusidic acid resistance encoding gene, *fusB and fusC* and only few samples tested carried the tiamulin resistance encoding gene *vga*(A) and *vgb*(A) or the multidrug resistant gene *cfr* and the gentamicin resistant gene *aacA-aphD*. Frey *et al*. (2013) found similar results in CoNS and suggested possible appearance of new resistance genes or mutation in the elongation factor G. The situation for gentamicin is somehow different depending on the breakpoint used. Isolates were considered all sensitive using the *S. aureus* breakpoint or all resistant using the CoNS breakpoint. However, no clear bimodal distribution of fusidic acid, tiamulin and gentamicin MICs was observed indicating a lower intrinsic susceptibility of *S. sciuri* to these antibiotics compared to the other staphylococcal species. Indeed, all isolates showed MIC's higher than the cut off value for these antimicrobials without bimodal distribution associated to wild and non-wild type. Further studies are necessary to confirm this since our collection of *S. sciuri* was selected on methicillin resistance and may not be representative enough.

Only one isolate was linezolid resistant accounting for about 1% of MRSS isolates which is in accordance with the meta-analysis of Gu *et al.* (2013) which reported linezolid resistance prevalence in non-*aureus* staphylococci (NaS) at 1.4% in hospital. Our strain was positive for the linezolid resistance encoding gene *cfr*, encoding cross-resistance to oxazolidinones, phenicols, streptogramin compounds, lincosamidins and pleuromutilins. The

dispersion of this gene should be closely monitored seen the broad spectrum of resistance this efflux pump gives.

Also for clindamycin, the EUCAST breakpoints do not seem to fit for *S. sciuri*. Here, there is a clear bimodal distribution and the proposed breakpoints for CoNS or *S. aureus* do not fit with this distribution. We found a resistance gene (*erm*(B), *erm(*C) or *cfr*) in eight strains of the "resistant" group (MIC>4) while none in the "susceptible" group (MIC: 0.5-2). Therefore, a better breakpoint would be > 2 giving a resistance percentage of 79.4%.

Breakpoints for quinupristin/dalfopristin and trimethoprim do not seem to fit either. However, the presence or absence of resistance genes could not help us in defining a clear breakpoint though for trimethoprim, a clear bimodal distribution can be seen.

Furthermore, while the strains in this study were isolated from healthy animals, *S. sciuri* harboured virulence genes also found in *S. aureus*. Since, some of these genes such as *sed*, *arcD* are located on mobile genetic elements (Bayles and Iandolo, 1989; Diep *et al*., 2004), these genes can be regarded as a pool for other staphylococci. Hence, *S. sciuri* might be an important reservoir of antimicrobial resistance and virulence genes for *S. aureus* and other staphylococci.

6.6. Conclusion

Contrary to what has been shown for MRSA, MRSS is a common bacterium in healthy chickens in Belgium. There is a major difference between the two ecological populations investigated, broilers and layers, indicating a separate epidemiology. Isolates were highly multi-resistant and carried a broad range of antimicrobial resistance and virulence genes that may be an important reservoir for other staphylococci such as *S. aureus*. Furthermore, this is, to our knowledge, the first study on the epidemiology of MRSS in healthy chickens.

6.7. Acknowledgments

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Chapitre 7 - Characterization of methicillin-resistant *Staphylococcus sciuri* isolates from industrially raised pigs, bovines and broiler chickens

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7.1. Abstract

This study aimed at assessing the epidemiology and genetic diversity of methicillin-resistant Staphylococcus sciuri (MRSS) from different farm animal species.

Nasal swabs were collected from 200 pigs, 100 dairy cows, 100 beef cows, 150 veal calves and 200 broilers. Colonies were isolated on selective media containing cefoxitin and the presence of the *mec*A gene was confirmed by PCR. Antimicrobial resistance was determined by microbroth dilution. Genetic diversity was assessed by pulsed field gel electrophoresis (PFGE) and resistance and virulence genes were detected by microarray.

The total MRSS prevalence at animal-level was estimated at 5.07% (n=71), varying from around 10% in broilers (n=20), dairy cows (n=10) and veal calves (n=20) to 6.5% in pigs (n=13), and 3.0% in beef cows (n=3). *mecA* was detected in all isolates. Staphylococcal cassette chromosome (SCC)*mec* type III and non-typeable SCC*mec* cassettes were the most frequent ones. Resistance against gentamicin, penicillin, tiamulin, clindamycin and quinupristin/dalfopristin was detected in more than 90% of isolates. Resistance to cefoxitin, fusidic acid and trimethoprim ranged between 78% and 87%. By using PFGE we were able to distinguish two major clusters.

All isolates tested by microarray carried the *mecA* gene and most showed erm and tet genes encoding macrolide-lincosamide and tetracycline resistance, respectively. Virulence genes were also detected including the immune-evasion factor encoding gene isa and the site specific deoxyribonuclease hsdS2.

This study shows that multi-resistant MRSS is carried by different farm animal types. Although some animals shared a same strain, PFGE showed different patterns indicating a large diversity among the MRSS isolates recovered. The absence of clusters associated with a certain animal species suggests low host specificity.

7.2. Introduction

Methicillin resistance (MR) in staphylococci, referring to resistance to all β-lactam antimicrobials, is an important concern in both human and veterinary medicine (Cohn *et al*., 2010). The most important gene encoding methicillin-resistance is the *mecA* gene, which is located on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito and Hiramatsu, 1998). *mecA* is widespread in *Staphylococcus aureus* and a variety of coagulase negative staphylococci (CoNS) species, from both human and animal origin (Wielders *et al*., 2002; Huber *et al*., 2011). In 2011 and until then, an unknown variant of *mecA*, called *mecC*, was described in human and bovines *population in the UK (García-Álvarez et al., 2011).* Species of the *S. sciuri* species group, including *S. lentus*, *S. vitulinus*, *S. fleurettii* and *S. stepanovicii*, are of special importance in relation to the origin and evolution of methicillin resistance genes. Indeed, *S. fleurettii* has been shown to carry the putative evolutionary ancestor of *mecA* (Tsubakishita *et al*., 2010). Furthermore, other *mecA* homologues appear to occur naturally in *S. sciuri* and *S. lentus* (Tulinski *et al*., 2012). Since these homologues do not always confer phenotypic resistance to methicillin (Monecke *et al*., 2012), *S. sciuri* are often considered as methicillin-susceptible (Couto *et al*., 1996). However, *S. sciuri* has been found to be methicillin resistant (Dakić *et al*., 2005; Nemeghaire *et al*., 2014a).

S. sciuri has long been considered as a non-pathogenic commensal bacterium, mostly recovered from skin and mucous membranes of warm blooded animals (Kloos *et al*., 1976; Adegoke, 1986). However, it is now also recognized as a potential pathogen responsible of various diseases such as mastitis, dermatitis and epidermitis in animals (Rahman *et al*., 2005; Hauschild & Wójcik, 2007; Chen *et al*., 2007) or endocarditis, wound infections, peritonitis, septic shock, and urinary tract infection in humans (Hedin & Widerström, 1998; Kolawole & Shittu, 1997; Wallet *et al*., 2000; Horii *et al*., 2001; Stepanović *et al*., 2003).

Methicillin resistant CoNS (MRCoNS) have been proposed to be a potential reservoir of SCC*mec* (Hanssen *et al*., 2006) for *S. aureus*. Furthermore, resistance genes and pathogenicity markers of *S. aureus* have been recovered in methicillin resistant *S. sciuri* (MRSS) isolates from chickens (Nemeghaire *et al*., 2014a). It is, though, important to investigate this in a more global context in order to have a general overview of the role of MRSS as a potential reservoir for resistance and virulence genes for methicillin-resistant *S. aureus* (MRSA).

The present study aimed at estimating the genetic diversity of MRSS isolated from healthy farm animals by means of SCC*mec* typing and Pulse Field Gel Electrophoresis (PFGE). Moreover, the role of MRSS as a potential resistance and virulence gene reservoir for other staphylococci was assessed through antimicrobial susceptibility testing and antimicrobial resistance and virulence genes detection.

7.3. Material and methods

7.3.1. Sampling and isolation methods

Nasal swab samples were collected in Belgium from randomly selected pig farms (n=10), dairy farms (n=10), beef farms (n=10), veal calf farms (n=15) and broiler farms (n=10). Twenty broilers were selected per farm resulting in a total of 200 broilers. On each pig farm, 10 animals of each represented age group were sampled, resulting in a total of 200 pigs (60 sows, 60 piglets and 80 fattening pigs). On each dairy, beef and veal calf farms, 10 animals were selected per age group resulting in a total of 100 dairy cows, 100 beef cows and 150 veal calves sampled. Veal calves were between three and 30 weeks of age; apart from seven heifers. All dairy cows were at least two years of age and similarly all beef cows were at least two years of age. Samples were enriched in brain heart infusion (BHI) with 7.5% NaCl at 37°C overnight. Isolation was performed on Columbia Agar (Oxoid,Germany)

supplemented with 5% sheep blood (BioMérieux, France) and 3.5 mg/l cefoxitin (Sigma-Aldrich, US), on ChromID *S. aureus* agar (BioMérieux, France), and on Columbia colistin-aztreonam agar supplemented with 5% sheep blood (Oxoid, France) as described before (Vanderhaeghen *et al*., 2012). Colonies were then purified on blood agar plates and visually inspected after 48 – 72h at 37°C in order to improve phenotypic identification.

Identification, mecA detection and SCCmec typing

DNA was extracted as previously described (Vanderhaeghen *et al*., 2010). The detection of the *mecA* and *mecC* gene, identification and SCC*mec* typing were performed as previously described (Vanderhaeghen *et al*., 2013). Results of SCC*mec* typing in pigs and bovines refer to previously published studies (Vanderhaeghen *et al*., 2012, Vanderhaeghen *et al*., 2013). Results of SCC*mec* in broilers were not previously published.

7.3.2. Macrorestriction-PFGE analysis

Whole genome DNA of isolates was prepared, digested by restriction enzyme SmaI and a PFGE was performed using a CHEF Mapper XA system (Bio-Rad Laboratories, United Kingdom). Plugs were prepared according to the protocol of Chung *et al*. (2012) with modifications. Plugs were prepared using BioRad Chef System plug molds and subjected to restriction with SmaI (Fermentas GmbH, Belgium) following the manufacturer's instructions. The electrophoresis conditions were 6 V/cm in 0.5x TBE (45mM Tris, 45mM boric acid, 1mM EDTA [pH 8]) at 11.3°C and runs lasted 23 h with switch times from 5s to 35s. PFGE profiles were compared using BioNumerics software (Version 6.6, Applied Maths, Belgium). A dendrogram was derived from Dice similarity indices based on the unweighted pair group method with arithmetic averages (UPGMA). *S. aureus* NCTC 8325 (National Collection of Type Cultures, United Kingdom) was included as control strain for PFGE analysis.

7.3.3. Antimicrobial susceptibility testing

Antimicrobial resistance was determined as previously described (Vanderhaeghen *et al*., 2013). The minimal inhibitory concentration (MIC) values were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-offs (ECOFF) for coagulase-negative staphylococci (CoNS). In the case there were no ECOFF available, ECOFF for *S. aureus* were used. Data from the EUCAST MIC distribution website was last accessed January 16, 2014 (http://www.eucast.org). When no ECOFF for CoNS or *S. aureus* was available, judgement of resistance was based on the frequency distribution of the MIC's as previously described (Butaye *et al*., 2003).

7.3.4. DNA microarray-based typing and detection of resistance and virulence genes

Twenty-two isolates were selected based on the different antimicrobial resistance phenotypes and PFGE profiles, in order to obtain the largest diversity. Microarray analysis was performed on these strains using the Identibac *S. aureus* Genotyping DNA Microarray (Alere Technologies GmbH, Köln, Germany) according to the manufacturer's instructions. The DNA microarray covers 333 oligonucleotide probes, detecting resistance and virulence genes. A full list including primer and probe sequences is available online (http://identibac.com/en/home.html).

7.4. Results

7.4.1. MRSS prevalence at animal level

Seventy-one MRSS were identified. Thirteen (6.50%, 95% CI [3.08-9.92]) of these isolates originated from pig farms, 25 (12.5%, 95% CI [7.92-17.08]) from broiler farms and 33 (9.43%, 95% CI [6.37-12.49]) were isolated from farms rearing bovines. Among these, 20 (13.33%, 95% CI [7.89-18.77) originated from veal calve farms, ten (10.0%, 95% CI [4.12-15.88]) from dairy farms and three (3.0%, 95% CI [0.0-6.34]) from beef farms (Table 18). MRSS isolates were recovered from seven different pig farms, eight dairy farms, two beef farms and two veal calf farms and eight broiler farms. The number of isolates recovered within the farms varied from one to 9 isolates per farms.

Table 18. Methicillin-resistant *Staphylococcus sciuri* prevalence and SCC*mec* distribution in different farms animals.

Categories (n samples)	n positive animals	Prevalence (%)	95% CI	SCC*mec* III (%)	SCC*mec* IIIA (%)	SCC*mec* NT (%)
Pigs (N=200)	13	6.50	[3.08-9.92]	61.54	7.69	30.77
Dairy cows (N=100)	10	10.00	[4.12-15.88]	60.00	35.00	5.00
Beef (N=100)	3	3.00	[0.00-6.34]	10.00	0.00	90.00
Veal calves (N=150)	20	13.33	[7.89-18.77]	33.33	0.00	66.67
Broilers (N=200)	25	12.50	[7.92-17.08]	92.00	8.00	0.00
Total (N=750)	71	9.47	[7.37-11.56]	63.38	14.08	22.54

CI, confidence interval; N, total number of MRSS isolated.

7.4.2. SCCmec and macrorestriction-PFGE analysis

Most isolates possessed the SCC*mec* type III, of which 45 (63.4%) were type III and 10 (14.1%) were type IIIA (Table 18). The remaining 16 (22.5%) isolates carried a non-typeable SCC*mec* cassette. Isolates from broilers carried mostly SCC*mec* type III type and type IIIA SCC*mec* with 23 (92.0%) and two (8.0%) isolates, respectively. Isolates from dairy cows and pigs carried mostly SCC*mec* type III with 12 (60.0%) and eight (61.5%) isolates, respectively and type IIIA SCC*mec* with seven (35.0%) and one (7.7%) isolate, respectively (Vanderhaeghen *et al*., 2012, Vanderhaeghen *et al*., 2013). Isolates from veal calves and beef carried mostly non-typeable cassettes with nine (90.0%) and two (66.7%) isolates, respectively (Vanderhaeghen *et al*., 2012, Vanderhaeghen *et al*., 2013).

Among the 71 MRSS isolates studied, five isolates did not show interpretable profiles in PFGE analysis and were thus considered non-typeable. PFGE analyses showed 37 different profiles grouped in two major clusters at 40% similarity (Figure 6). The first cluster was composed of 25 isolates grouped in fourteen profiles. This cluster was composed of nine (36.0%) isolates from veal calves, eight (32.0%) from broilers, five (20.0%) from pigs and three (12.0%) from beef. This cluster counted also 15 (60.0%) SCC*mec* type III, seven (28.0%) type IIIA and three (12.0%) non-typeable SCC*mec*. The first cluster could be divided in two subclusters (1a and 1b) at 50% similarity. The second cluster was composed of 41 isolates grouped in 23 profiles. This cluster was composed of 16 (39.0%) isolates from broilers, nine (22.0%) from dairy cows, eight (19.5%) from pigs and eight (19.5%) from veal calves. This cluster counted also 24 (58.5%) SCC*mec* type III and 15 (36.6%) non-typeable SCC*mec*. The second cluster could be divided in two subclusters (2a and 2b) at 50% similarity. Isolates from a same farm were scattered among both clusters and in different clades of the dendrogram..

7.4.3. Antimicrobial resistance

All isolates were resistant to gentamicin. Most isolates were resistant to penicillin (98.6%, 95% CI [98.3 – 98.9]), clindamycin (98.6%, 95% CI [98.3 – 98.9]), tiamulin (98.6%, 95% CI [98.3 – 98.9]), quinupristin/dalfopristin (91.5%, 95% CI [90.8 – 92.3]), cefoxitin (87.3%, 95% CI [86.4 – 88.2]), fusidic acid (87.3%, 95% CI [86.4 – 88.2]) and trimethoprim (78.9%, 95% CI [77.7 – 80.0]). More than half of the isolates were resistant to tetracycline (62.0%, 95% CI [60.6 – 63.3]), mupirocin (56.3%, 95% CI [55.0 – 57.7]) and erythromycin (52.1%, 95% CI [50.7 – 53.5]). A little more than 25% of the isolates were additionally resistant to streptomycin (28.2%, 95% CI [26.9 – 29.4]) and around ten percent to kanamycin (14.1%, 95% CI [13.1 – 15.0]) and ciprofloxacin (9.86%, 95% CI [9.04 – 10.7]). Few isolates were resistant to chloramphenicol (8.45%, 95% CI [7.68 – 9.22]), sulfamethoxazole (4.23%, 95% CI [3.67 – 4.78]), linezolid (1.41%, 95% CI [1.08 –1.73]) and rifampicin (1.41%, 95% CI [1.08 –1.73]). None were resistant to vancomycin (Table 19). All isolates were multi-resistant and showed resistance to at least four antimicrobials and to a mean of 7.4 antimicrobials ranging from 6.76 for broiler farms to 8.8 for dairy farms.

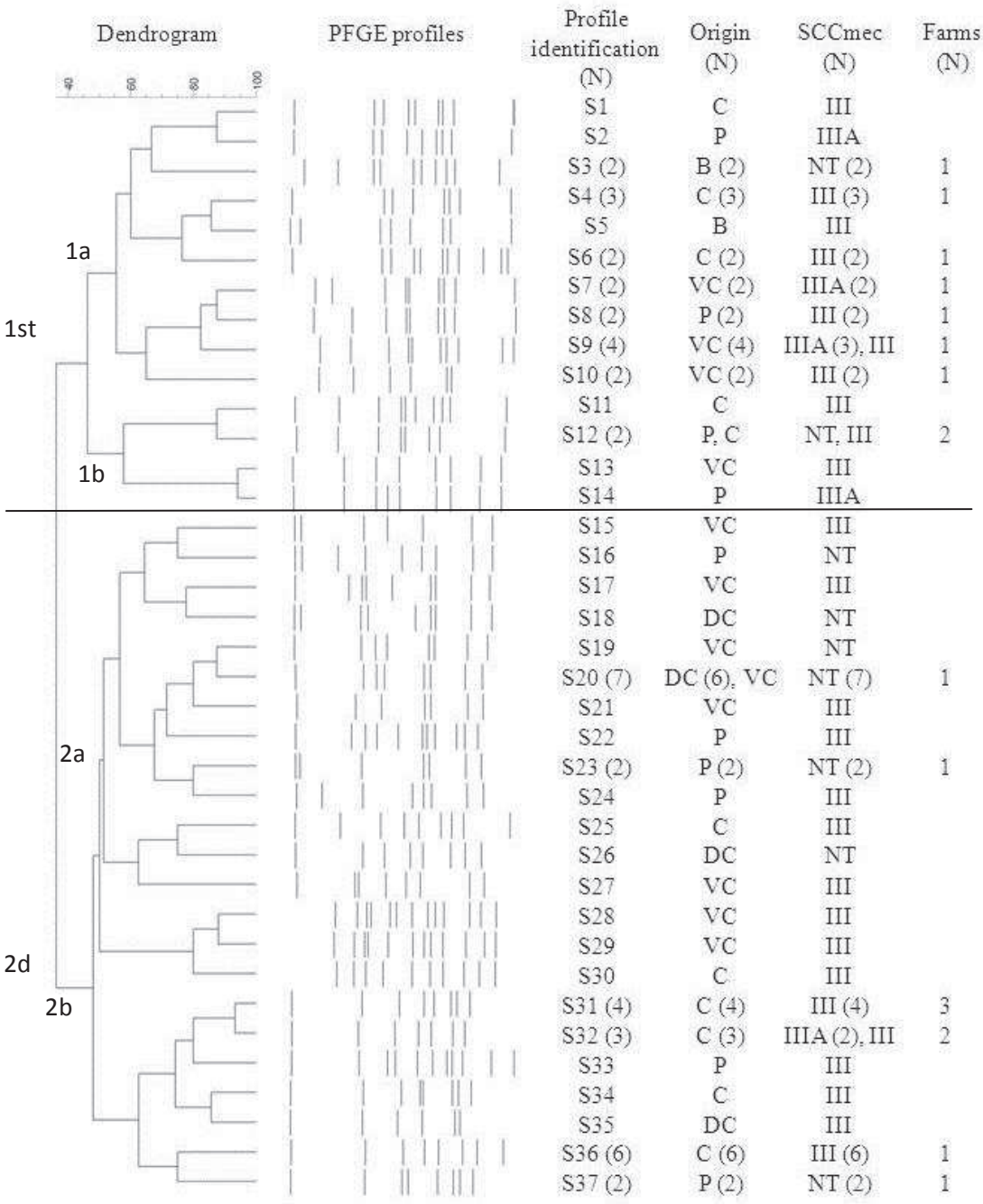

Figure 6. Dendrogram derived from Dice similarity indices based on the unweighted pair group method with arithmetic averages (UPGMA). The horizontal line shows the separation of the two major clusters. 1st, first cluster; 2d, second cluster. N, number of isolates when more than one; n, number of farm in a cluster when more than one isolate; NT, non-typeable; S, SmaI-PFGE profile.

Table 19. MIC distribution of methicillin-resistant *S. sciuri* from farm animals

Antimicrobials	% of isolates with MIC (mg/l) of																%R
	<=0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
CHL									60.56	30.99	0.00	1.41	5.63	1.41			8.45
CIP						12.68	57.75	19.72	0.00	0.00	9.86						9.86
CLI					1.41	0.00	2.82	46.48	1.41	47.89	0.00						98.59
ERY						47.89	0.00	0.00	2.82	0.00	0.00	49.30					52.11
FOX							0.00	1.41	0.00	11.27	16.90	47.89	22.54				87.32
FUS							12.68	0.00	14.08	69.01	4.23						87.32
GEN								90.14	2.82	5.63	1.41	0.00					100.0
KAN										83.10	2.82	7.04	5.63	1.41			14.08
LZD								67.61	30.99	0.00	1.41						1.41
MUP							36.62	7.04	53.52	0.00	0.00	0.00	0.00	0.00	0.00	2.82	56.34
PEN				1.41	1.41	4.23	21.13	35.21	36.62								98.59
RIF	98.59	0.00	0.00	0.00	0.00	1.41											1.41
SMX													88.73	7.04	4.23	0.00	4.23
STR									56.34	7.04	8.45	7.04	21.13				28.17
SYN							1.41	7.04	63.38	26.76	1.41						91.55
TET							38.03	0.00	0.00	0.00	0.00	0.00	61.97				61.97
TIA							1.41	0.00	0.00	1.41	97.18						98.59
TMP									21.13	47.89	5.63	1.41	0.00	23.94			78.87
VAN								94.37	5.63	0.00	0.00	0.00					0.00

MIC, minimal inhibitory concentration; R, resistance; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, cefoxitin FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; LZD, linezolid; MUP, mupirocin; PEN, penicillin; RIF, rifampicin; SMX, sulfamethoxazole; STR, streptomycin; SYN, quinupristin/dalfopristin; TET, tetracyclin; TIA, tiamulin; TMP, trimethoprim; VAN,vancomycin.

Empty boxes indicate the concentration values that were not tested. Values in grey boxes indicate MIC higher than the concentration tested.

The bold lines indicate epidemiological cut-off values for *S. aureus*. The pointed lines indicate epidemiological cut-off values for coagulase-negative staphylococci while different of the *S. aureus*. MIC values were interpreted using the EUCAST clinical breakpoints /epidemiological cut-offs.

7.4.4. Microarray typing for resistance and virulence gene detection

Twenty-two isolates with different PFGE profiles at 80% similarity and different antimicrobial resistance profiles were selected for microarray analysis. All isolates carried *mecA*, together with other SCC*mec* genes such as *mecR*, *ΔmecR*, *mecI* and the glycerophosphoryl diester phosphodiesterase, associated with *mecA* gene, *ugpQ*. Among the five isolates carrying a non-typeable SCC*mec* tested with the microarray, two were negative for the *ccrB-1* and one of these two was also negative for *ccrA-3* and *ccrB-3* while all other isolates were positive for those genes. Both SCC*mec* type IIIA isolates carried *ccrA* and *ccrC* genes. Several resistance genes were found in the collection. All erythromycin resistant isolates tested carried at least one macrolides, lincosamides, streptogramin B (MLS_B) resistance gene of the *erm* family. Six isolates carried *erm*(A), nine carried *erm*(B) and four carried *erm*(C). Resistance to clindamycin and quinupristin/dalfopristin was also high, probably due to the erm genes since no isolates carried typical *S. aureus* lincosamide, pleuromutilins and streptogramin A resistance genes *vga*(A) and *vgB*(A). Only five isolates showed the aminoglycoside adenyl-/phosphotransferase encoding gene *aacA-aphD*. Six kanamycin resistant and three kanamycin sensitive isolates were positive for the *aadD* aminoglycoside resistance gene. Among the eighteen tetracycline-resistant strains, five carried *tet*(K) and sixteen *tet*(M). All chloramphenicol resistant and one chloramphenicol sensitive isolates carried the chloramphenicol acetyltransferase (*cat*) gene. Ten isolates carried the lincosaminide nucleotidyltransferase, *lnu*(A) though the lincomycin resistant phenotype could not be determined since this antimicrobial was not included in the susceptibility testing. The penicillin-ampicillin resistance operon *bla* (*blaZ, blaI,* and *blaR*) was not detected. Five isolates carried the *qacC* efflux pump gene encoding resistance against quaternary ammonium disinfectants.

Additionally to the resistance genes recovered, isolates were positive for several virulence genes. The immune-evasion factor (*isaB*), the staphylococcal superantigen-like proteins (*ssl10/set4*), the protease (*sspP*) and the site specific deoxyribonuclease (*hdsS2* and *hdsSx*) genes were detected in more than 70% of the isolates. More than 25% of the isolates carried the leukocidin encoding gene (*lukS*), the fibronectin-binding protein A (*fnbA*), immune-evasion factor (*isdA*) and the bone sialoprotein-binding protein (*bbp*). The biofilm associated gene (*bap*), the putative transporter (*ImrP*), the bone sialoprotein-binding protein C (*sdrC*) and the *S. aureus* surface protein G (*sasG*) encoding genes were found in around 20% of the isolates. Other virulence genes such as the staphylococcal superantigene-like (*setB2* and *ssl03/set8*), the putative membrane protein (*hlIII*), the enterotoxin encoding gene (*sed*), the arginine catabolic mobile element (ACME) locus (*arcD-SCC*) and capsule (*capK1*) encoding genes were found in only one isolate.

7.5. Discussion

S. sciuri is an ancestral species among the genus *Staphylococcus* (Kloos *et al.,* 1976). Long considered as a commensal species (Adegoke, 1986), several clinical cases have now been reported in both human and veterinary medicine (Nemeghaire *et al.* 2014b). However, little is known on its epidemiology and genetic diversity in healthy animals. This study aimed to estimate the prevalence of MRSS in different healthy farm animals and to determine its genetic diversity in order to assess its potential role as a reservoir for virulence and resistance genes for other staphylococci such as *S. aureus*.

We found 71 MRSS leading to a prevalence ranging from 3% for beef to 10% for dairy cows and veal calves. The prevalence in broilers (12.5%) is quite low compared to a former study on MRSS in Belgium which was estimated at farm level at around 30% (Nemeghaire *et al*. 2014a). However, the study of MRSS in poultry reported estimated

between-herd prevalence while the present study reports prevalence at animal level. It has also to be noted that these two studies used different isolation methods. Other studies showed the presence of MRSS in different farms animals such as chickens (Kawano *et al.,* 1996), horses (Yasuda *et al.,* 2002; Busscher *et al.,* 2006) and dogs (Bagcigil *et al.,* 2007) though no accurate prevalence could be estimated.

SCC*mec* analysis showed a higher prevalence of type III cassette which accounted for more than 60% of the SCC*mec* recovered. Non-typeable SCC*mec* were found in more than 20% of the isolates. This is in accordance with Zhang *et al.* (2009) who found SCC*mec* type III predominant MRCoS from animal origin. The distribution of SCC*mec* among the different animals showed that while broiler, dairy and pig farms harboured mostly SCC*mec* type III, veal calves and beef carried mostly a non-typeable cassette. The hypothesis of a possible specific distribution of SCC*mec* among animals has already been proposed in a previous study where broiler chickens showed mostly the type III SCC*mec* while layer chickens showed a high prevalence of a non-typeable SCC*mec* (Nemeghaire *et al.* 2014a). Though no *ccr* complex could be detected by the PCR described by Kondo *et al.* (2007) in the non-typeable SCC*mec*, only one of these isolates was negative for all *ccr* tested in the microarray. This was also observed in the study of MRSS in poultry in Belgium (Nemeghaire *et al.* 2014a). This supports the hypothesis of the carriage of a *ccrA* element with an aberrant sequence that was proposed in this latter study. This high prevalence of non-typeable SCC*mec* also confirms the potential divergence of *ccr* and *mec* complexes in *S. sciuri* as suggested by Urushibara *et al.* (2011).

PFGE with SmaI restriction was used here to assess diversification among isolates since this method has been shown to have a high discriminative power in *S. aureus* (Murchan *et al.,* 2003). The 66 typable isolates were grouped in 37 different unique profiles. Some isolates were recovered from different animals at the same farms. However, some of these

isolates were separated among the different clusters observed. The different animal origins were also scattered in different clusters. This indicates high diversity among the farms sampled and a low host specificity. These results differ from those of the study on poultry in Belgium (Nemeghaire *et al.* 2014a) since in the latter study, broiler and layer *S. sciuri* isolates were clearly separated in two different clusters. Nonetheless, SCC*mec* types seemed to be separately distributed among the two major clusters. Indeed, while all isolates carrying the type IIIA SCC*mec* were grouped in the first cluster, most non-typeable isolates were grouped in the second cluster. This indicates the relative low mobility of the SCC*mec* element.

Unexpectedly, not all isolates showed phenotypic resistance to penicillin and cefoxitin in the micro-broth dilution tests, despite the use of selective media for MRSS isolation and the presence of the *mecA* gene in all isolates. However, since ECOFF are not available for those two antimicrobials in CoNS, this method might not be reliable in this case (http://www.eucast.org/). Additionally to this, the EUCAST breakpoints for *S. aureus* and for CoNS for clindamycin and trimethoprim do not seem to fit with the observed distribution. Indeed a clear bimodal distribution was seen for clindamycin with a "resistant group" (MIC > 4) and a "susceptible group" (MIC < 2). However, we found resistance genes (*ermA*, *ermB* or *ermC*) in all strains from those both groups. We are thus not able to propose a better breakpoint for clindamycin. On the other hand, for trimethoprim, a clear bimodal distribution was seen with a "resistant group" with a MIC around 4 mg/l and a "susceptible group" with a MIC around 32 mg/l. Since the trimethoprim resistance genes, *dfrS1* (*dfrA*), were not detected, the resistance may be due to other *dfr* genes such as *dfrD* or *dfrG* which were not included in the *S. aureus* genotyping kit and have previously been detected in members of the *S. sciuri* species group (Schnellmann *et al.,* 2006).

All isolates were resistant to gentamicin using ECOFF breakpoint for CoNS. However, only isolates with a MIC ranging between 2 mg/l and 4 mg/l were positive for the

gentamicin resistance gene *aacA-aphD*. Since, the cut off value to determine wild type and non-wild type for this antimicrobial is ≤ 2mg/l for *S. aureus* while it is ≤ 1mg/l for CoNS, this indicates that gentamicin ECOFF breakpoint for *S. aureus* is more accurate than that of CoNS for *S. sciuri*. It is also to be noted here that EUST plates used in this study do not allow detection of MIC lower than 1 mg/l for gentamicin. It is thus not possible to differentiate between isolates with a MIC equal or lower to 1 mg/l while ECOFF breakpoint for CoNS is ≤0.5 mg/l. Resistance was very high for tiamulin and fusidic acid though no resistance genes associated to those antimicrobials could be detected. This situation has previously been described by Frey *et al.* (2013) who suggested possible appearance of new resistance genes or mutations in the elongation factor G. Few isolates were resistant to linezolid, which is in accordance with the meta-analysis of Gu *et al.* (2013) which reported around 1.4% of the CoNS in hospital resistant to linezolid. In the previous study on MRSS in poultry (Nemeghaire *et al.* 2014a), we found the *cfr* gene associated to phenotypic linezolid resistance However, in this study we did not detect this gene, indicating an alternative mechanism of resistance.

Several of our *S. sciuri* isolates harboured virulence genes such as *sed* and *arcD* which are located on mobile genetic elements (Bayles & Iandolo, 1989; Diep *et al.,* 2004). These isolates may thus be regarded as a source of virulence genes for other staphylococci the livestock associated MRSA ST398, which carries currently few virulence genes (Argudín *et al*., 2011; Jamrozy *et al.,* 2012). The presence of resistance and virulence genes similar to those found in *S. aureus* enhances the hypothesis that *S. sciuri* might be an important reservoir of these genes.

7.6. Acknowledgment

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Part IV – General Discussion

After the introduction of penicillin in the 1940s, β-lactamase mediated resistance has spread fast (Chamber, 1988). To counteract this type of resistance, penicillinase stable β-lactam antibiotics such as methicillin were developed. However, a new resistance mechanism quickly emerged, named methicillin resistance and this is well known in *S. aureus* resulting in methicillin resistant *S. aureus* (MRSA). Though methicillin resistance (MR) was recognized in coagulase negative staphylococci (CoNS) even before the first report on MRSA in humans (Stewart, 1961), more attention is usually given to MRSA than to MRCoNS. After the emergence of MRSA in hospitals, an increased incidence of infections caused by MRSA in the community was noticed since mid-1990's (Vandenesch *et al*., 2003). In addition to this, an increase in infections with MRCoNS species was recognized in hospitals, though, these are considered less virulent than MRSA (von Eiff *et al*., 2002). Also in the community, MRCoNS have frequently been described as commensals (Barbier *et al*., 2010). In veterinary medicine, however, methicillin resistance in staphylococci was rarely reported until the 1990s. From this time onwards, reports of epidemic MRSA outbreaks (Scott *et al*., 1988), single infections (Cefai *et al*., 1994) and nasal carriage in companion animals (Manian *et al*., 2003) increased. In parallel, MRCoNS such as *S. sciuri*, *S. epidermidis* and *S. saprophyticus* were increasingly reported from healthy animals (Kawano *et al*., 1996; Yasuda *et al.*, 2000), and other CoNS species were isolated from cases of bovine mastitis (Myllys *et al*., 1998; Tenhagen *et al*., 2006; Piessens *et al*., 2012). Finally, round 2005, the first MRSA in pigs, and from humans in contact with these animals, were reported (Voss *et al.*, 2005; Armand-Lefevre *et al*., 2005). Through the increased interest of MRSA in animals, it was noticed that this specific MRSA was not only present in pigs but also in other livestock animals, and therefore it was named Livestock-associated (LA)-MRSA. Its virulence potential was, however, low.

The origin of the *mecA* gene coding for methicillin resistance in staphylococci is thought to be a homologous gene that is present on the chromosome of members of the *S.*

sciuri species group (Wu *et al*., 1996; Wu *et al*., 1998, Monecke *et al*., 2012). Analyses of this *mecA* gene showed that this homologous could be regarded as the precursor of the acquired *mecA* gene located on the SCC*mec* carried by MRSA (Tsubakashita *et al*., 2010). It is thus thought that the *mecA* gene was mobilised on a mobile genetic element (MGE). It was demonstrated that the LA-MRSA, belonging to the clonal complex (CC)398, originated from human methicillin sensitive *S. aureus* (MSSA), and became adapted to livestock where it acquired the staphylococcal cassette chromosome (SCC)*mec* element. As methicillin resistance was not present in *S. aureus* from livestock, the *mecA* seemed likely to have originated from CoNS (Price *et al*., 2012). Although the mechanisms and direction of SCC*mec* transfer are still poorly understood, CoNS are thought to be potential reservoirs for antimicrobial resistance for other species such as *S. aureus* (Hanssen and Sollid, 2006).

In Belgium in 2007, MRSA prevalence was investigated in pigs and estimated at around 68% of the farms being positive for the typical LA-MRSA CC398 (Crombé *et al*., 2012). This survey highlighted variations in prevalence depending on the age group, with piglets showing a higher prevalence than sows or fattening pigs. Others studies also showed the presence of MRSA in poultry, although at a low approximate carriage rate in broilers and at around 13% of broiler farms harbouring CC398 MRSA (Nemati *et al*., 2008; Persoons *et al*., 2009). However, these studies were quite limited in the number of farms sampled and isolation methods other than the internationally comparable method, recommended by the EFSA, were used.

Therefore, in order to have comparable results between the different surveillances performed in the European Union over the different animal species and to have a more accurate view on the prevalence, we carried out a large survey in poultry in Belgium in 2011 (**Chapter 4**). In this survey, the prevalence in poultry was shown to be much lower than that in pigs and also lower than what was noticed in the former limited studies (Nemati *et al*.,

2008; Persoons *et al*., 2009). However, in the isolation method we used in this surveillance, swabs were pooled per farm and this might play a role in this lower prevalence. Indeed, it has been shown that a culture of pooled swabs may have a lower sensitivity than culture from separate swabs (Grmek-Kosnik *et al*., 2005).

A high prevalence had been found in veal calves in the Netherlands (Graveland *et al*., 2010) and in cases of mastitis in Belgium and Germany (Vanderhaeghen *et al*., 2012; Feβler *et al*., 2010). In the present thesis, we investigated the nasal carriage of MRSA in Belgian bovines. We found an overall prevalence of approximately 20% (**Chapter 5**), although there was a major difference between veal calves on the one hand, and dairy cows and meat producing cattle on the other hand, confirming previous results (Graveland *et al*., 2010; Vandendriessche *et al*., 2013). The reason for this difference between age groups and rearing practices is not clear yet and needs further investigation. It has been shown that stress may alter the bacterial microbiota. Indeed, stress induces an increase of blood cortisol level which has been linked with excretion of *Salmonella* by pigs (Verbrugghe *et al*., 2011). Similar results were found in veal calves after moving them from farm through feed yard (Corrier *et al*., 1990). It might thus be that stress conditions such as moving, regrouping or separation of the calves from their mothers increases stress in young animals, modifying their microbial carriage. Antimicrobial use has also been shown to be very intensive in veal calves and much higher than in conventional dairy and beef cattle (Pardon *et al*., 2012). Additionally to this, the use of antimicrobials has been shown to be a risk factor for LA-MRSA carriage in veal calves (Bos *et al*., 2012). To reduce antimicrobial resistance spread in veal calf farming, several recommendations have been published. These recommendations propose sparsely using antimicrobials for prophylaxis and to use quinolones and 3d and 4th generation cephalosporins for treatment only when no other antimicrobials are effective. It has also been advised to avoid prophylactic use of antimicrobials and replace them by targeted

metaphylactic treatment at the appearance of clinical symptoms (http://www.amcra.be/nl/rundvee).

Since *S. sciuri* is known for its ubiquitous presence, in warm blooded animals and in the environment, we carried out an in depth study on the prevalence of methicillin resistant *S. sciuri* (MRSS) in poultry. MRSS may be an interesting indicator bacterial species for monitoring prevalence of antimicrobial resistance in staphylococci. Additionally, since *S. sciuri* can be efficiently isolated using the same methodology as *S. aureus*, this allows estimating an accurate prevalence from a same sampling and isolation methodology. Our studies showed that approximately 30% of the poultry farms (**Chapter 6**) and 12.5% of the broilers sampled (**Chapter 7)** were positive for MRSS. This prevalence was substantially higher than what was found for MRSA in the same population. However, since methicillin sensitive *S. aureus* and *S. sciuri* were not screened in this study, we cannot state that *S. sciuri* is more common in this population than *S. aureus*.

It is remarkable that MRSS were detected in around 30% of the egg producing farms. This high prevalence does not seem to be correlated to the use of antimicrobials since these are sparsely used in layer production to avoid veterinary drug residues in chicken eggs (Goetting *et al*., 2011; Persoons *et al*., 2012). It can however not be excluded that some of the layers that carried MRSS had been treated with β-lactam antibiotics during their youth.

Hospital acquired (HA)-MRSA is known to have a clonal population structure and this is also the case for LA-MRSA. Indeed, it has been shown that MRSA recovered from livestock mainly belong to CC398 (Armand-Lefevre *et al*., 2005). Furthermore, the clonality of LA-MRSA is supported by *spa* typing since geographic clustering of *spa* types among CC398 MRSA in Europe has been shown (Köck *et al*., 2009; Gomez-Sanz *et al*., 2010). In our studies, CC398 isolates mainly carried *spa* type t011 or closely related *spa* types (**Chapter 4** and **5**).

We also found other non-LA-MRSA in poultry and bovines. Indeed, three isolates from chickens and two isolates from dairy cows were of sequence type (ST)239 which is considered as a HA-MRSA. This ST seems to be uncommon in humans in Belgium and is found mainly in Eastern Europe, Asia and the USA (Cha *et al*., 2005; Smyth *et al*., 2009; Harris *et al*., 2010). This clone has also been recovered from dogs in Canada (Lin and Davies, 2007) and in China (Zhang *et al*., 2011). Interestingly, all three MRSA ST239 isolates from poultry showed the same resistance profile. Although pulsed field gel electrophoresis (PFGE) analysis has not been performed, this result may indicate that these isolates belong to the same strain. MRSA ST239 isolates from bovines also showed an identical resistance profile but which was different from that of poultry isolates. The origin of the presence of MRSA ST239 in poultry and bovines remains to be elucidated in a specific research focussing on characterisation of these isolates. We need to decipher whether the isolates found here are similar/identical to those found in humans. Secondly, since the clone has been found in poultry and bovines (and also in 2013 in pigs, unpublished results, CODA-CERVA), it should be figured out whether ST239 MRSA lacks host specificity as is the case for the CC398 clone. We may characterise the strains by whole genome sequencing, given the fact that this technique has become cheaper and is more easily accessible than before. Furthermore, we have no information on the MRSA status of the personnel. Such epidemiological data would bring more information on the possible relatedness of herds and possible transmission of this clone between farms.

In addition to this, the human associated clone MRSA ST8 was found in a beef rearing farm. MRSA ST8 has previously been found in hospitals in Belgium (Denis *et al*., 2004) and is considered as an archaic clone which is widespread both in the hospital environment and in the community (Deurenberg *et al*., 2006). This clone has also been recovered from horses and people working in contact with horses in Canada. In the Canadian study, it was suggested that

this human epidemic clone became adapted to horses (Weese *et al*., 2010). Emergence of human MRSA in animals has been documented in several studies though in most cases, these clones were not able to maintain themselves in animal populations as it was the case for LA-MRSA CC398 (Devries *et al*., 1975).

The origin of ST8 and ST239 in our studies is unknown. Since these two STs are known as human pathogens, a transmission of virulence genes to LA-MRSA might lead to important public health concerns. Indeed, although at present MRSA ST398 is considered less virulent than other STs (Argudín *et al*., 2011), its wide dispersion in animals would make it an important pathogen in case of virulence transmission to this clone.

In LA-MRSA CC398, most SCC*mec* cassettes are of type IV or V (Vanderhaeghen *et al*., 2010; Price *et al*, 2012). Also in our studies, LA-MRSA harboured mainly SCC*mec* types IV or V (**chapter 4, chapter 5**). These cassettes were also frequently recovered from MRSA isolates from pigs (de Neeling *et al*., 2007; Crombé *et al*., 2012). SCC*mec* types IV and V are small elements considered as having a higher mobility than larger SCC*mec* elements (Hanssen and Solid, 2006). These two cassettes have also been shown not to contain antimicrobial resistance genes other than the *mecA* gene (Okuma *et al*., 2002), except for the *czrC* gene encoding cadmium and zinc resistance in type V (Cavaco *et al*., 2010). Since metal-containing compounds are frequently used in pigs in the prevention of gastrointestinal disease or as a feed supplement, it is not surprising to find this cassette frequently in LA-MRSA. We found SCC*mec* type III in MRSA ST8 and ST239 (**Chapter 4** and **5**). This SCC*mec* is usually recovered from HA-MRSA tough it has also been found in community associated (CA)-MRSA, albeit to a lesser extent (Deurenberg and Stobberingh, 2008). In contrast to type IV and V SCC*mec*, this cassette contains transposons encoding macrolide-lincosamide-streptogramin (MLS) and cadmium (*cad*) resistance as well as tetracycline (*tet*) and mercury resistance (Ito *et al*., 1999; Hanssen and Solid, 2006).

Contrary to what we found in LA-MRSA, our MRSS isolates contained mainly SCC*mec* types III and IIIA. In MRCoNS, type III SCC*mec* is commonly recovered from human isolates (Mombach *et al*., 2007; Zong *et al*., 2011) as well as from animal isolates (Zhang *et al*., 2009; Vandehaeghen *et al*., 2012; Vanderhaeghen *et al*., 2013). Since type III SCC*mec* was found in MRSA ST239, isolated from chickens, as well as in MRSS isolates from pigs, bovines and chickens, this supports the hypothesis of a possible SCC*mec* exchange between MRSA and MRCoNS such as *S. sciuri*. Since SCC*mec* type III was found in a wide diversity of MRSS of different animal origin and since the genetic background of MRSS is diverse, we postulated that this cassette might already be present for a long time in these farm animals. Furthermore, the type III cassette was found in human MRSA long before the discovery of the LA-MRSA (Deurenberg *et al*., 1985). It may thus have been harboured by MRSS even before the spread of MR in CC398 *S. aureus* in livestock animals. Further analyses of type III SCC*mec* in MRSA and in MRSS are necessary in order to determine the homology of this cassette in these two species. This would allow a better understanding of the possible exchange of this cassette among MRSA and MRSS of human and animal origin.

In MRCoNS, non-typeable SCC*mec* are often found in human and animal isolates (Barbier *et al*., 2010; Tulinski *et al*., 2012; Shittu *et al*., 2012; Vanderhaeghen *et al*., 2013). In our study, we also frequently found non-typeable SCC*mec* isolates in which no *ccr* element could be detected using the M-PCR described by Kondo *et al*. (2007) (**chapter 6** and **7**). This is in agreement with the results of Hanssen *et al*. (2004; 2005), who also described the presence of the *mec* locus in the absence of known *ccr* genes in MRSA and MRCoNS. This might be explained by: unrecognized *ccr* types; high mutation rate in the binding sequence of the *ccr* primers; homologous recombination between *ccr* genes generating new non-detectable ccr complexes; deletion of the *ccr* genes or independent transmission of the *mecA* (Hanssen and Solid, 2006). The last hypothesis is rather unlikely since integration sites are necessary

for recombinase allowing integration of foreign DNA into the staphylococcal chromosome to recognise its target. We also found non-typeable SCC*mec* isolates among LA-MRSA from bovines (**chapter 7**), and they have been described before in Belgian pigs (Crombé *et al*, 2012). The presence of SCC*mec* variants and of non-typeable cassettes indicates a rather diverse SCC*mec* reservoir in animal staphylococci. Furthermore, the high prevalence of non-typeable SCC*mec* confirms the high diversity of *ccr* and *mec* elements in *S. sciuri*, as suggested by Urushibara *et al*. (2011). Further studies on these non-typeable SCC*mec* may bring insight in the evolution and plasticity of these elements which are still poorly understood nowadays. This may allow us to propose intervention strategies to limit the spread of SCC*mec* elements.

Presently, we cannot investigate the population structure of *S. sciuri* by MLST since no typing scheme has been developed as has been for *S. aureus* or *S. epidermidis*. However, other typing methods such as macrorestriction analysis followed by PFGE can be used to assess the clonal relatedness between *S. sciuri* isolates (Couto *et al*., 2000). Using this fingerprinting method, we demonstrated the presence of two main clades in chickens (**chapter 6**). A clear distinction could be made between strains from broilers and layers. This may be explained by the fact that those two populations are raised separately with little spill over. These two breeds are the result of a specific hybrid crossings leading to different morphologies (www.fao.org). Indeed, while broiler chickens are genetically selected for their fast growth and high muscle production, laying hens are selected for their intensified egg production. Since broilers and layers are raised separately, it is not that surprising to see different MRSS populations associated to these breeds. Furthermore, these two MRSS groups were also shown to be associated to a different SCC*mec* distribution. Indeed, while broiler MRSS were shown to carry mainly SCC*mec* type III, layer isolates carried mostly a non-

typeable cassette. This would suggest a possible environmental niche associated to a particular SCC*mec*.

This distinction between populations could not be seen among the different other farm animals (**chapter 7**). SCC*mec* types also seemed to be scattered over the different clades. This indicates a high diversity of MRSS in Belgian livestock and also suggests that either MRSS is highly capable of transmission to different animal species and therefore, has quite low host specificity, or that the bacteria evolve quite fast and that the methicillin resistance has been in the population for quite a while. However, it should also be noted that the total number of MRSS isolates in each animal category was rather low. Further studies are necessary to determine the specific epidemiological features, including ecological niches, of this species.

Presence of antimicrobial resistance in CoNS has been poorly investigated and EUCAST breakpoints for CoNS are not always available. Additionally, our results showed that *S. aureus* cut-off values were not always applicable to *S. sciuri*. Indeed, in our MRSS isolates, *S. aureus* EUCAST breakpoints for antimicrobials such as clindamycin and trimethoprim do not seem to fit with the observed distributions. Also, testing the bacteria for the presence of known resistance genes does not always bring a solution since tiamulin resistance, typically more prevalent in MRSS compared to MRSA, was not associated with any known tiamulin resistance genes. New breakpoints should then be proposed in order to accurately differentiate wild type from non-wild type phenotypes for each CoNS species.

MRSA and MRSS are found in farm animals and they might share a common gene pool. In our study, we found several resistance genes in MRSS isolates that were also present in MRSA. These resistance genes are also widely detected in other coagulase positive and coagulase negative staphylococci of animal origin (Wendlandt *et al*., 2013).

In our studies, the presence of different resistance genes coding for the same resistance phenotype, such as *tet*(M) and *tet*(K) was shown. Since these genes are frequently located on

MGEs, such as transposons or plasmids, they may have been acquired separately (Wendlandt *et al*., 2013). Furthermore, it has to be noted that *tet*(M) carrying transposons can also carry additional resistance genes such as *erm*(B), a*phA3*, *sat4* or *aadE* (Lyon and Skurray, 1987; Cochetti *et al*., 2008). Indeed, several of our MRSS isolates carried both *tet*(M) and *erm*(B). Of notice is the presence of the multidrug resistance gene, *cfr*, encoding cross-resistance to all phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A in MRSS isolates from poultry. This gene was first identified in CoNS of animal origin (Schwarz *et al*., 2000; Kehrenberg and Schwarz, 2006), but is not frequently found in MRSA of animal or human origin (Kehrenberg *et al*., 2009). This gene is not only important with regard to the penta-resistance phenotype it encodes, but to date it is also the only known gene that confers transferable resistance to oxazolidinones, which are last resort antimicrobial agents in human medicine (Witte and Cuny, 2011).

An interesting difference was noted between MRSS and MRSA isolates in our studies. Indeed, while MRSS were frequently resistant to fusidic acid (**Chapter 6** and **7**), as also shown in other studies (Shittu *et al*., 2004; Frey *et al*., 2013), resistance to this antimicrobial was much lower in bovine (**Chapter 5**) and chicken (**Chapter 4**) MRSA isolates. Fusidic acid was shown to be effective in the treatment of staphylococcal infections such as impetigo due to coagulase positive and negative staphylococci, regardless whether they are methicillin resistant or susceptible (Verbist, 1990; Koning *et al*., 2002). However, increase of fusidic acid prescription led to an increase of frequency of fusidic acid resistance in CA-*S. aureus* in the UK and northern Europe (Brown and Wise, 2002; Dobbie and Gray, 2004). Until now, fusidic acid resistance in *S. aureus* remains low (Howden and Grayson, 2005; O'Neill *et al*., 2007). The difference in the prevalence fusidic acid resistance between *S. aureus* and *S. sciuri* may be due to a different resistance mechanism. Indeed, while resistance in MRSA is linked to *fus* genes, *S. sciuri* isolates are commonly found to be negative for the presence of acquired

fusidic acid resistance genes (Castanheira *et al*., 2010; Frey *et al*., 2013). It has been proposed that yet to be described resistance genes or mutations in elongation factor G might be implicated in *S. sciuri* fusidic acid resistance (Frey *et al*., 2013). In our studies as well, no known fusidic acid resistance genes could be detected in MRSS.

LA-MRSA typically contains few virulence genes, and as a consequence, little pathology is associated with it. It should be noted nonetheless, that LA-MRSA CC398 has been isolated from mastitis in bovines (Feβler *et al*., 2010; Vanderhaeghen *et al*., 2012) and that both methicillin resistant and methicillin susceptible CC398 strains have been isolated from infections in turkeys (Argudin *et al*., 2013). Given the fact LA-MRSA is extremely capable of acquiring foreign DNA, it is important to also investigate the virulence determinants in other staphylococci sharing the same environment with *S. aureus*. In our studies, few virulence genes were detected in MRSS isolates compared to MRSA isolates. On the other hand, some virulence genes that were detected in our MRSS isolates could not be found in MRSA. Unlike *S. sciuri*, *S. aureus* is considered more capable of causing a wide range of diseases in humans and animals. One of the most important virulence factors in both *S. aureus* and CoNS is their ability to produce biofilms, protecting the bacteria against antibiotics and host immune responses. This biofilm formation is regulated by the *ica* operon, encoding intercellular adhesion proteins, and *bap* genes encoding surface proteins improving bacterial adhesion and biofilm associated protein. Although the *ica* operon was frequently found in *S. aureus* from poultry (Argudín *et al*., 2013; Nemati *et al*., 2014), we could not detect it in MRSS nor in our MRSA isolates. The *bap* gene that has previously been shown to enhance intramammary adherence and has been associated with bovine mastitis isolates (Cucarella *et al.*, 2001), was detected in some MRSS isolates while it could not be detected in MRSA. Furthermore, one of the first stages in the pathogenesis of *S. aureus* infections is adhesion mediated by adhesins called “microbial surface components recognizing adhesive

matrix molecules" (MSCRAMMs). These molecules mediate adherence to host tissues containing collagen, fibronectin and fibrinogen (Gordon and Lowy, 2008). Several MRSS isolates were found to carry at least one of these adhesion factors such as *bbp* and *fnbA*. In humans, these genes have been found to be implicated in *S. aureus* endovascular infection as well as in bone and joint infections (Gordon *et al*., 2008). In poultry, *fnbA* has commonly been detected in MSSA and MRSA isolated from healthy and diseased animals while *bbp* has rarely been found (Argudín *et al*., 2013; Nemati *et al*., 2014).

Other virulence factors of *S. aureus* are the leukocidins which have a function in maintenance of the bacteria in the host by leukocyte destruction. Leukocidin encoding genes, such as *lukS*, were detected in most of our MRSA and MRSS isolates and have been shown to be frequently present in staphylococci of animal origin (Monecke *et al.*, 2007; Argudín *et al*., 2013; Nemati *et al*., 2014). Additionally, the capsule encoding gene (*capK1*), encoding a capsular polysaccharide (CP), protecting bacteria from phagocytosis, was found in few MRSS isolates. While most bovine MRSA carried the *cap5* gene, some MRSS isolates from poultry and veal calves were positive for the *capK1* gene. This CP type 1 has been shown to be an important and powerful virulence factor (Lee *et al*., 1987, Luong *et al*., 2002). The fact that some MRSA and MRSS isolates in our studies carried similar virulence genes and that these may be located on MGEs and may likewise be transferred between bacterial strains, indicates that *S. sciuri* may also act as a reservoir of virulence genes for *S. aureus.* Since we found MRSA clones associated with humans and in particular the HA-MRSA, in poultry and bovines, transmission of these MGE located virulence genes to LA-MRSA is theoretically possible and should be monitored. Since these LA-MRSA are widely distributed, such transmission would lead to important concerns for human and animal healthcare.

In conclusion, we showed the importance of MRSA and MRSS as a reservoir for SCC*mec* and other antimicrobial resistance genes and for virulence genes in farm animals. It

is therefore important to pay attention to these commensals and to consider them in a risk analysis for human health. Many aspects of SCC*mec* mobility are still unknown since its transmission is poorly understood. Although horizontal transfer of SCC*mec* is supported by epidemiological data, its in vitro transfer has not been described yet (Hanssen and Solid, 2006). Nonetheless, the variety of SC*Cmec* elements as well as the high frequency of non-typeable cassettes in diverse clones of CoNS including *S. sciuri*, demonstrate the high plasticity of the SCC*mec* element and indicate frequent gene exchange. Detailed analysis of these non-typeable elements should bring more information on their structure.

Part V - References

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Summary - Samenvatting

Summary

This doctorate aimed at determining the epidemiology and genetic diversity of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant *Staphylococcus sciuri* (MRSS) in order to understand whether *S. sciuri* might play a role as reservoir for antimicrobial resistance genes and virulence genes for other staphylococci. *S. aureus* is recognized as a facultative pathogen and, among other things, has been frequently associated with nosocomial infections in hospitals and mastitis in bovines. *S. sciuri,* on the other hand, is one of the most ancient species in the genus *Staphylococcus* and is mainly considered as a harmless environmental species though it has also been recovered, albeit rarely, from infections in humans and animals.

In this doctorate, we estimated the prevalence of MRSA and methicillin-resistant *Staphylococcus sciuri* (MRSS) in Belgian poultry herds in 2011 and in Belgian bovine herds in 2012. In the meantime, two isolation methods were compared. One of the isolation methods was proposed by the EFSA and used two enrichment broths. The alternative method was very similar though only one enrichment broth was used. Although no significant difference could be found between the estimated prevalence of both methods tested during the survey in poultry, the method recommended by EFSA was found less sensitive than the alternative method. Based on this, it was proposed to use the method with the highest sensitivity in population where a low MRSA prevalence is expected. This also allowed saving one day during the isolation procedure.

We found an in-herd MRSA prevalence of 0.8% in poultry and of 18.7% in bovines. The estimated prevalence of MRSA in bovines was much higher than that in poultry and was also dependent on the age of the animals. While no significant difference was demonstrated between the prevalence in broiler and laying hens, the prevalence of MRSA in dairy (9.93%) and beef farms (10.16%) was significantly lower than in veal calf farms (46.15%).

Another variable in the prevalence of methicillin resistance among staphylococci is the staphylococcal species. Indeed, the in-herd MRSS prevalence was estimated at 31.0% in poultry which was significantly higher than that of MRSA. Also the prevalence of MRSS at animal level differed between both animal species and age groups. MRSS prevalence at animal level was estimated at approximately 10% in broiler chickens, dairy cows and veal calves which was higher than the estimated prevalence in pigs (6.5%) and beef cows (3%).

The genetic diversity of both MRSA and MRSS was determined. While there are well defined molecular tools available for *S. aureus* characterization, this is not the case for *S. sciuri*. s*pa*-typing and multi locus sequence typing (MLST) showed the presence of a broad range of different MRSA types in bovines although the livestock-associated (LA)-MRSA ST398 was the most common. Unexpectedly, ST239 MRSA has been found both in bovines and in poultry. Since this type is a common hospital acquired (HA)-MRSA, this support the hypothesis of a spill-over and spill-back between animals and humans. For *S. scuri,* our studies could rely only on staphylococcal cassette chromosome (SCC)*mec* typing and pulsed field gel electrophoresis (PFGE) for subtyping of the population. SCC*mec* typing showed marked differences between MRSA and MRSS. While MRSA mostly harboured SCC*mec* type IV and V, MRSS carried mostly SCC*mec* type III and non-typable SCC*mec.* In poultry non-typeable SCC*mec* were mainly found in MRSS of laying hens, while type III was found mainly in MRSS of broilers. PFGE also showed that *S. sciuri* isolates from laying hens differed from that of broiler chickens. This clear association of some *S. sciuri* cluster with a particular species group was not visible in another study using MRSS isolated from various farm animals. This supports the hypothesis of an environmental origin of MRSS and eventually also *S. sciuri*.

We found also that both *S. aureus* and *S. sciuri* are often multi-resistant to antimicrobials. There were, however quite some differences in the resistance profiles. While

MRSA were most frequently resistant to tetracycline and trimethoprim, MRSS were more frequently resistant to fusidic acid, tiamulin and quinupristin/dalfopristin. The prevalence of trimethoprim and clindamycin resistance was high in both species while frequency of resistance to ciprofloxacin and chloramphenicol was low. All isolates were susceptible to vancomycin. We found also that EUCAST cut-off values were not always accurate to make the distinction between wild- and non-wild types in MRSS. For antibiotics such as clindamycin and trimethoprim, new cut off values for *S. sciuri* were proposed. The resistance genes found in these two staphylococcal species were frequent the same indicating a common source of resistance genes.

Finally, we demonstrated the presence of several virulence genes in both MRSA and MRSS from different animal origins. The virulence genes found are involved in biofilm formation, encoding leukocidins, or capsular polysaccharides. Since some of these genes are located on mobile genetic elements, they may be a potential reservoir of virulence genes for other staphylococci.

In this PhD, we have shown the importance of MRSA and MRSS in farm animals as reservoir of antimicrobial resistance genes as well as virulence genes. The frequent occurrence of non-typeable SCC*mec* cassettes in various MRSS isolates is illustrative of the plasticity of the SCC*mec* element. As antimicrobial resistance and virulence genes may be located on mobile genetic elements one should consider these two species as a potential threat to public health

Samenvatting

In dit doctoraat hebben we de epidemiologie en de genetische diversiteit van methicilline resistente *Staphylococcus aureus* (MRSA) en methicilline resistente *Staphylococcus sciuri* (MRSS) Bestudeerd. *S. aureus* is een facultatief pathogene kiem, die verschillende aandoeningen kan veroorzaken bij de mens en diverse diersoorten. Bij mensen wordt deze kiemsoort onder andere gevreesd als oorzaak van nosocomiale infecties en bij runderen is het een belangrijke oorzaak van mastitis. *S. sciuri* is daarentegen één van de oudste species binnen het genus *Staphylococcus* en wordt beschouwd als een onschuldige, in het milieu voorkomende bacterie. Sporadisch wordt deze kiem evenwel geïsoleerd uit diverse letsels bij mensen en dieren.

We hebben de prevalentie van MRSA bepaald in neusstalen van Belgisch pluimvee in 2011 en in nasale swabs van gezonde runderen in 2012. Tijdens dit onderzoek werden twee isolatiemethoden vergeleken. De eerste isolatiemethode maakte gebruik van twee aanrijkingen en is de door het European Food Safety Authority (EFSA) voorgestelde methode. Deze wordt echter als niet erg gevoelig beschouwd. Onze alternatieve methode is vergelijkbaar met de eerste, maar waarbij slechts één aanrijking gebeurt. Hoewel er geen significant verschil was in de geschatte prevalentie voor beide methoden, was de methode aanbevolen door de EFSA numeriek minder gevoelig dan de alternatieve methode. Op basis hiervan werd voorgesteld om de methode met de hoogste gevoeligheid te gebruiken in populatie waarbij een laag MRSA prevalentie is verwacht. Dit geeft bovendien een tijdsbesparing van een dag in de isolatie procedure.

Bij pluimvee werden dezelfde stalen ook gebruikt voor het schatten van de prevalentie van methicilline resistente *Staphylococcus sciuri* (MRSS).

De geschatte prevalentie van MRSA bij runderen (18.7%) was veel hoger dan bij pluimvee (0.8%). De prevalentie is ook afhankelijk van de leeftijd van de dieren. Terwijl er

geen significant verschil kon aangetoond worden tussen de prevalentie bij vleeskuikens en legdieren, was de prevalentie van MRSA bij melk- (9.93%) en vleesvee (10.16%) significant lager dan bij vleeskalveren (46.15%).

Een andere variabele in het voorkomen van methicilline resistentie bij stafylokokken is het stafylokokken species. Inderdaad de MRSS prevalentie bij pluimvee (31.0%) was beduidend hoger dan de prevalentie van MRSA. Ook in de prevalentie van MRSS werden verschillen tussen zowel dierspecies als leeftijdsgroep waargenomen. Bij vleeskuikens, melkvee en vleeskalveren werd een hogere prevalentie (ongeveer 10%) waargenomen dan bij varkens (6.5%) en vleesvee (3%).

De genetische diversiteit van zowel MRSA als MRSS werd geanalyseerd. In tegenstelling tot *S. sciuri*, zijn er goed gedefinieerde moleculaire tools beschikbaar om *S. aureus* te karakteriseren, waardoor de populatiestructuur en spreiding van de verschillende clones beter geanalyseerd kunnen worden. Via *spa*-typering en multi locus sequentie typering (MLST) konden we aantonen dat er bij runderen een breed scala aan verschillende MRSA-subtypes aanwezig waren, hoewel de dier-geassocieerde (LA)-MRSA ST398 de meest voorkomende was. Tegen de verwachting in, werden er ook niet-ST398 MRSA gevonden en dit zowel bij runderen als bij pluimvee. Het gevonden type, ST239, is een hospitaal geassocieerd (HA)-MRSA sequentie type. De hypothese van een “spill-over” en “spill–back” tussen dieren en mensen wordt daarmee bevestigd. Voor subtypering van de *S. sciuri* populatie dienden we te vertrouwen op staphylococcal cassette chromosome (SCC)*mec* typering en pulsed field gel elektroforese (PFGE). De SCC*mec* types van MRSA en MRSS waren verschillend, terwijl MRSA meestal het SCC*mec* type IV en V bezaten, bezaten MRSS meestal het SCC*mec* type III of niet typeerbare SCC*mec* types (NT-SCC*mec*). Bij pluimvee werden de niet-typeerbare SCC*mec* voornamelijk gevonden bij *S. sciuri* van legdieren, terwijl het type III voornamelijk werd teruggevonden bij *S. sciuri* van vleeskuikens. Ook PFGE

toonde aan dat de *S. sciuri* isolaten van legdieren verschillenden van deze van vleeskuikens. Deze duidelijke associatie van een bepaalde *S. sciuri* cluster met een bepaalde diergroep, was niet zichtbaar in een andere studie waarbij MRSS die geïsoleerd werden bij diverse landbouwhuisdieren vergeleken werden via SCC*mec* typering en PFGE. Inderdaad, in deze laatste studie, waren isolaten van dezelfde oorsprong (kippen, varkens of runderen) en isolaten van dezelfde bedrijven verspreid over verschillende clades van het dendrogram en was de diversiteit aldus veel hoger dan in de vorige studie.

Zowel *S. aureus* als *S. sciuri* vertoonden een hoge graad van multiresistentie tegenover antimicrobiële middelen en kunnen als dusdanig als een reservoir voor antimicrobiële resistentiegenen beschouwd worden. Er werden wel verschillen teruggevonden in de resistentieprofielen tussen deze twee stafylokokkensoorten. MRSA waren meestal resistent tegenover tetracyclines. Voor MRSS kwam resistentie tegenover fusidinezuur, tiamuline en quinupristine/dalfopristine vaak voor terwijl ciprofloxacine en chloramfenicol resistentie weinig gedetecteerd werd. Resistentie tegenover trimethoprim en clindamycine kwam frequent voor bij beide species. Alle isolaten waren gevoelig voor vancomycine. Er dient opgemerkt te worden dat de EUCAST cut-off-waarden niet altijd accuraat waren om het onderscheid tussen wild- en niet-wild-type stammen binnen MRSS te maken. Voor trimethoprim en clindamycine werden dan ook andere breekpunten voorgesteld voor *S. sciuri*. De resistentiegenen waren voor deze twee stafylokokken species frequent dezelfde wat duidt op een gemeenschappelijke pool van resistentiegenen.

In een laatste studie, konden we meerdere virulentiegenen aantonen in zowel MRSA als MRSS van verschillende oorsprong. Er werden virulentiegenen die betrokken zijn bij biofilmvorming, productie van leukocidines en kapselpolysacchariden gedetecteerd in zowel MRSA als MRSS. Aangezien deze genen eveneens op mobiele genetische elementen kunnen

liggen, dienen we ook *S. sciuri* als een potentieel reservoir voor virulentiegenen te beschouwen.

In dit doctoraat hebben we het belang van MRSA en MRSS bij landbouwhuisdieren aangetoond als reservoir van zowel antimicrobiële resistentiegenen als virulentiegenen. Het frequent voorkomen van niet typeerbare SCC*mec* cassettes in diverse MRSS clones is illustratief voor de plasticiteit van het SCC*mec* element. Aangezien antimicrobiële resistentiegenen en virulentiegenen op mobiele genetische elementen gelokaliseerd kunnen zijn, dient men deze twee kiemen als een potentieel gevaar voor de volksgezondheid te beschouwen.

About the author

Stéphanie Nemeghaire was born in Brussels on the 21st of July 1984. After the end of her study at secondary school in Economy-Arts at the Institut des Sœurs de Notre-Dame in Anderlecht, she obtained in 2009 the diploma of Master in Organisms biology and Ecology; with a specialization in Eco-Ethology at the Université Libre de Bruxelles (ULB). After finishing her study with distinction, she worked for a short period in a bookstore.

She started her PhD in February 2011 at the Department of Pathology, Bacteriology and Poultry Diseases at the Veterinary Faculty of the University of Ghent and at the Veterinary and Agrochemical Research centre (CODA-CERVA) under the supervision of Pr. Dr. Patrick Butaye, Pr. Dr. Freddy Haesebrouck and Dr. María de los Ángeles Argudín. The topic of her PhD is the "Epidemiological investigation into the possible exchange of SCC*mec* between staphylococci in different ecosystems" which was financed by the European LA-MRSA EMIDA project. During her thesis, she also had the opportunity to visit the laboratory of ANSES in Lyon during a short term mission financed by the MED-VET-Net association.

Stéphanie Nemeghaire is author and co-author of several scientific publications and reports. She also participated to national and international congresses and supervised students during her thesis research.

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"I have arrived. I am home. My destination is in each step"

Thich Nhat Hanh