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Molecular epidemiology of livestock-associated methicillin-resistant *Staphylococcus aureus* in the Belgian pork production chain

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List of abbreviations

AB	Antimicrobial agent
ATCC	American Type Culture Collection
BE	Belgium
BELVET-SAC	Belgian Veterinary Surveillance of Antimicrobial Consumption
BHI	Brain-heart infusion broth
bp	Base pairs
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
cfu	Colony forming units
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CODA-CERVA	Centrum voor Onderzoek in Diergeneeskunde en Agrochemie
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
D / DI	Simpson’s index of diversity
DE	Germany
DGZ	Animal Health Care Flanders
DK	Denmark
DNA	Desoxyribo Nucleic Acid
dUTP	Deoxyuridine Triphosphate
ECDC	European Centre for Disease prevention and Control
EFSA	European Food Safety Agency
EU	European union
FASFC	Federal Agency for the Safety of the Food Chain
FAVV	Federaal Agentschap voor de veiligheid van de voedselketen
FR	France
H	Herd
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
HIV	Human Immunodeficiency virus
IL	Illinois
ILVO	Institute for Agricultural and Fisheries Research

List of abbreviations

IWT	Institute for the Promotion of Innovation by Science and Technology in Flanders
JP	Japan
KATHO	Catholic University College Southwest-Flanders
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
MGE	Mobile Genetic Element
MHB	Mueller Hinton Broth
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
MLVA	Multilocus Variable-number tandem-repeat Analysis
MN	Minnesota
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MST	Minimum Spanning Tree
NA	Not available
NB	Nutrient Broth
NC	North-Carolina
NL	The Netherlands
NP	Not present
NS	Not significant
NT	Non-typeable
Orf	Open Reading Frame
OR	Odds ratio
ORSAB	Oxacillin Resistance Screening Agar Base
PBP	Penicillin-binding protein
PBP2a	Penicillin-binding protein 2a
PPD	Personal Protective Devices
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PVL	Panton-Valentine leukocidin
rDNA	Ribosomal DNA

List of abbreviations

SCC	Staphylococcal Cassette Chromosome
SCC*mec*	Staphylococcal Cassette Chromosome *mec*A
SIRU	Staphylococcal interspersed repeat units
SPF	Specific Pathogen Free
ST	Sequence type
TSA	Tryptone Soy Agar
UK	United Kingdom
UPGMA	Unweighted Pair Group Method using Averages
US / USA	United States of America
VAR	Veterinary and Agrochemical Research Centre
WGS	Whole genome sequencing
WI	Winsconsin
Wt/vol	Weight-volume percent

Preface

The first description of methicillin-resistant *Staphylococcus aureus* (MRSA) occurred only few years after the first clinical use of the antibiotic methicillin. Until 2000, MRSA was only found in the hospital environment (i.e. hospital-associated MRSA, HA-MRSA) and the community (i.e. community-associated MRSA, CA-MRSA). In 2005, a new MRSA type was isolated from livestock animals and especially pigs. The high prevalence of this livestock-associated MRSA (LA-MRSA) in pigs, the rare occurrence of LA-MRSA infections in pigs, the isolation of LA-MRSA from humans working with pigs and its ability to take in virulence and resistance genes, led to concerns about the transmissibility of this MRSA type to the general human population.

In 2009, a Belgian co-operation of partners (Institute for Agricultural and Fisheries Research (ILVO); Catholic University College of South-West-Flanders (KATHO) and the Department of Bacterial diseases, Veterinary and Agrochemical Research Centre (VAR)) started a project on LA-MRSA in pig farming to reduce the amount of LA-MRSA on pig farms. The focus of the present PhD work was situated on studying the epidemiology of LA-MRSA throughout the Belgian pig production chain by means of molecular tools.

First, an overview of the literature is given. The three MRSA types and their characteristics are described. In addition, the current knowledge on LA-MRSA transmission from the pig production chain to the general human population is discussed. The isolation and molecular characterization methods for LA-MRSA are given. Last, the Belgian pig production chain is described.

Second, epidemiological studies were performed to gain more insights into i) the possible differences in MRSA presence and genetic diversity of different animal species present at the farm (chapter III); ii) the colonization age of piglets and the effect of the mother sow and the environment on this age (chapter IVa); iii) the genetic diversity throughout time (chapter IVb) and iv) the MRSA prevalence and genetic diversity on pig carcasses and pork (chapter Va and Vb).

Third, all results obtained during the experimental studies are discussed and conclusions were drawn. In addition, future work is assessed.

Chapter I. Review of the literature

1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive and coagulase-positive facultative pathogenic bacterium that is classified within the genus *Staphylococcus* in the family of *Staphylococcaceae*. *S. aureus* is able to adapt to various hosts and the majority of clones is host specific. It is naturally carried by humans, various animal species and most food producing animals, such as pigs and cattle.

S. aureus has some remarkable features that enable this bacterium to survive in various hosts. First, a large genetic diversity amongst *S. aureus* isolates is observed. This is due to the presence of more than 700 core variable (CV) genes besides a highly conserved backbone of core genes (Dancer, 2008; Stefani et al., 2012). Second, *S. aureus* possesses a wide variety of virulence and quorum sensing mechanisms, which enable the bacterium to survive the immune reactions of the host and to cause several infections (Dancer, 2008; Tong et al., 2011; Moellering, 2012). Moreover, *S. aureus* has the ability to acquire new genes, (e.g. antimicrobial resistance genes) mainly through mobile genetic elements, enabling the bacterium to adapt to new environmental conditions or selective pressures (Stefani et al., 2012).

1.1 Clinical manifestations in humans

S. aureus is a bacterium that is worldwide spread amongst humans. Approximately half of the human population is a non-carrier of *S. aureus* and the other half an asymptomatic carrier: around 20% being a persistent carrier and around 30% being an intermittent carrier (Wertheim et al., 2005; Chambers and DeLeo, 2009). Upon persistent carriage, higher loads of one *S. aureus* clone were detected, whereas upon intermittent carriage, lower loads of various *S. aureus* clones were detected (Wertheim et al., 2005). Nasal carriage of *S. aureus* has been identified as a risk factor for subsequent *S. aureus* infection.

This bacterium may cause various human infections ranging from mild infections (for example: furuncles and abscesses) to more severe and more invasive infections as pneumonia, septicemia, endocarditis and septic arthritis. In addition, the toxins of *S. aureus* are capable of causing diseases such as food poisoning, the toxic-shock syndrome or staphylococcal scalded skin syndrome. Approximatley 40% of the mortality from nosocomial infections worldwide is caused by *S. aureus* (Cosgrove et al., 2003; Wertheim et al., 2005; Durai et al., 2010).

1.2 Clinical manifestations in animals

In animals, various pathogenic *Staphylococcus* species are known to cause infections: *S. aureus*, *Staphylococcus hyicus* and the *Staphylococcus intermedius* species group. *S. aureus* causes disease in cattle (mastitis), small ruminants (mastitis), poultry (joint infections, osteomyelitis, dermatitis, arthritis and septicemia), rabbits (mastitis, pustular and exudative dermatitis, subcutaneous abscesses, conjunctivitis, purulent rhinitis and pododermatitis), pigs (septicemia and exudative epidermitis), horses (dermatitis and cellulitis) and other animal species. *S. hyicus* attributes to exudative epidermitis, sporadic joint infections or cystitis in pigs and exudative skin infections of cattle, horses and poultry. The *S. intermedius* species group consists of *Staphylococcus pseudintermedius*, *S. intermedius* and *Staphylococcus delphini*. The species of this group are responsible for septicemia in ducks and pigeons and dermatitis in mink and horses. *S. pseudintermedius* is the predominant species causing skin infections in dogs and *S. delphini* causes skin diseases in horses (Hermans et al., 2010; Vanderhaeghen et al., 2010a; Haesebrouck, 2012).

2 Methicillin-resistant *Staphylococcus aureus*

2.1 Mechanism of methicillin resistance

Methicillin resistance is due to the presence of adapted penicillin-binding proteins (PBP), playing an active role in the cell wall synthesis. In non-resistant isolates, methicillin will bind to these proteins, which results in the disruption of the synthesis of the peptidoglycan layer. This disruption emphasizes the formation of pores in the cell wall, resulting in a disturbed osmotic pressure. Due to the inflow of fluids, cell death occurs. The cell wall of MRSA isolates contains both regular PBP and PBP2a. PBP2a has a decreased affinity for β-lactam antibiotics, resulting in cell wall synthesis in the presence of β-lactam antibiotics and even of the second generation antibiotics cephalosporins and carbapenems (Hartman and Tomasz, 1984). The genes that encode PBP2a are the *mec*A and *mec*C gene which are located on the Staphylococcal Cassette Chromosome *mec* or SCC*mec*, a mobile genetic element. *Mec*A is only expressed in presence of β-lactam antibiotics and is regulated by *mec*I and *mec*R1, which are both divergently transcribed. MecR1 is a transmembrane β-lactam sensing signal transducer and MecI represses the *mec*I-*mec*R operon and subsequently *mec*A in absence of antibiotics. When β-lactam antibiotics bind to MecR1, this transducer is cleaved autocatalytically. Subsequently, the cytoplasmatic end of MecR1, the metalloprotease

domain, becomes active and cleaves MecI. This causes transcription of the *mec*A operon and successively the production of PBP2a (Figure I-1) (Berger-Bachi and Rohrer, 2002; Deurenberg and Stobberingh, 2008).

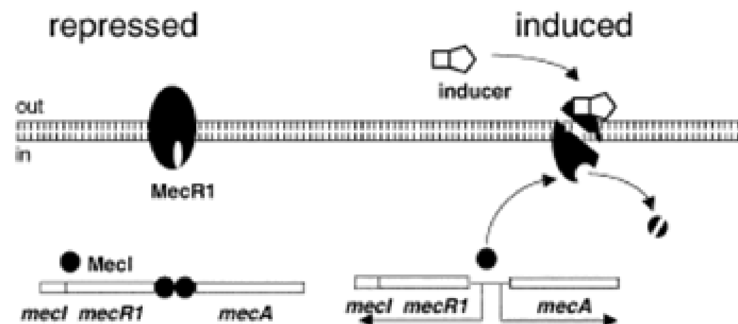

Figure I-1 Overview of the *mec*I-*mec*R1/*mec*A complex when repressed and when induced by a β-lactam antibiotic. The black dot represents MecI that represses the transcription of the *mec*I-*mec*R1/*mec*A operon in the absence of a β-lactam antibiotic. In the presence of a β-lactam antibiotic, MecI is cleaved, resulting in the transcription of the operon (Berger-Bachi and Rohrer, 2002).

The SCC*mec* cassette consists of different units of which four are located on all cassettes (Figure I-2). First, there is an insertion sequence IS431, which allows insertion of the cassette into the genome at a unique site in OrfX, the bacterial chromosomal attachment site *attB*SCC with sequence TATGATANGCNTCTCC (Cuny and Witte, 2005). The second component is the *mec* complex which consists of the *mec*A gene with *mec*I and *mec*R1 or a truncated *mec*R1 complex (Δ*mec*R1). Third, cassette chromosome recombinase (*ccr*) genes are present, which allow integration into and excision of SCC*mec* from the genome. Last, three non-essential or joining (J) regions are present: one between the right junction and the *ccr* genes (J1), one between the *ccr* genes and the *mec* complex (J2) and one from the *mec* complex to OrfX (J3) (Ito et al., 1999; Hanssen and Sollid, 2006; Deurenberg and Stobberingh, 2008).

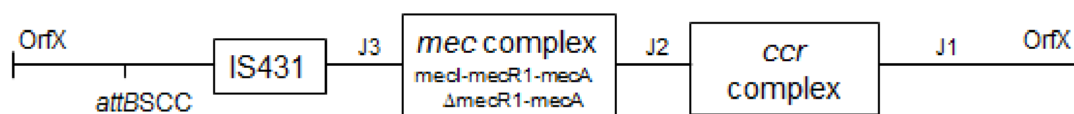

Figure I-2 Basic structure of the SCC*mec* cassette. Four components are always present: Insertion sequence (IS) 431, *mec* complex (*mec*I, *mec*R1 and *mec*A or Δ*mec*R1-*mec*A), *ccr* complex and three joining regions (J1-3). *attB*SCC is the unique site in which the cassette integrates in the genome.

The SCC*mec* type is determined based on the structure of the *mec* complex and the presence of the *ccr* genes. There are five *mec* and eight *ccr* complexes with specific characteristics. At present, eleven major SCC*mec* cassette types are known of which an overview is shown in Table I-1 (www.sccmec.org). Subtyping of the types is based on the junkyard regions and a subtype is indicated with a lower case letter (e.g. subtype IVa). Each type can carry additional genes, such as antibiotic resistance genes (Hansen and Sollid, 2006; Deurenberg and Stobberingh, 2008).

Table I-1 Summary of the eleven reported SCC*mec* cassettes (www.sccmec.org; Ito et al., 2004; Li et al., 2011).

SCC*mec* type	*ccr* gene complex[a]	*mec* gene complex
I	1 (A1-B1)	B (IS431-*mec*A-Δ*mec*R1-IS1272)
II	2 (A2-B2)	A (IS431-*mec*A-mecR1-*mec*I)
III	3 (A3-B3)	A (IS431-*mec*A-mecR1-*mec*I)
IV	2 (A2-B2)	B (IS431-*mec*A-Δ*mec*R1-IS1272)
IV	2 (A2-B2) & 5 (C1)	B (IS431-*mec*A-Δ*mec*R1-IS1272)
V	5 (C1)	C2 (IS431-*mec*A-Δ*mec*R1-IS431)[b]
V	5 (C1) & 5 (C1)	C2 (IS431-*mec*A-Δ*mec*R1-IS431)[b]
VI	4 (A4-B4)	B (IS431-*mec*A-Δ*mec*R1-IS1272)
VII	5 (C1)	C1 (IS431-*mec*A-Δ*mec*R1-IS431)[c]
VIII	4 (A4-B4)	A (IS431-*mec*A-mecR1-*mec*I)
IX	1 (A1-B1)	C2 (IS431-*mec*A-Δ*mec*R1-IS431)[b]
X	7 (A1-B6)	C1 (IS431-*mec*A-Δ*mec*R1-IS431)[c]
XI	8 (A1-B3)	E (*bla*Z-*mec*A$_{LGA251}$-*mec*R1$_{LGA251}$-*mec*I$_{LGA251}$)[d]

[a] the gene combination is shown between brackets
[b] two IS431s are arranged in the opposite direction
[c] two IS431s are arranged in the same direction
[d] *mec*A$_{LGA251}$ has been renamed in *mec*C

An MRSA strain is obtained when an MSSA strain acquires a SCC*mec* cassette through horizontal gene transfer. Many studies have been conducted to determine the origin of *mec*A and the SCC*mec* cassette. It is hypothesized that a strain of *Staphylococcus fleurettii* is the ancestral source of *mec*A. *S. fleuretti* has a detectable methicillin resistance. Sequencing of the *mec*A gene revealed a high degree of homology with the *mec*A gene of MRSA (Couto et al., 2003). In *Staphylococcus hominis*, a SCC was found without the *mec* determinant (Katayama et al., 2003). Tsubakishita and collegues (2010) speculate that the *mec*A gene and the SCC cassette recombined within a MSSA isolate under the selective pressure of β-lactam antibiotics. From this isolate on, the cassette transferred to other human isolates.

2.2 Evolution of MRSA

The evolution methicillin-resistant *Staphylococcus aureus* (MRSA) can be divided into different waves, which correspond to certain time periods. After the first use of methicillin in the clinical environment, MRSA strains were found in the hospitals (end 1950 – 1970) where highly successful lineages emerged around the 1970s until the mid-1980s. Early 1990s, the first MRSA strains emerged in the community independent of the hospital environments. Around 2005, a distinct clone of MRSA, MRSA ST398 or livestock-associated MRSA, was described in livestock animals for the first time. Nowadays, MRSA ST398 can also be found in humans and their environment independent of livestock animals (Figure I-3).

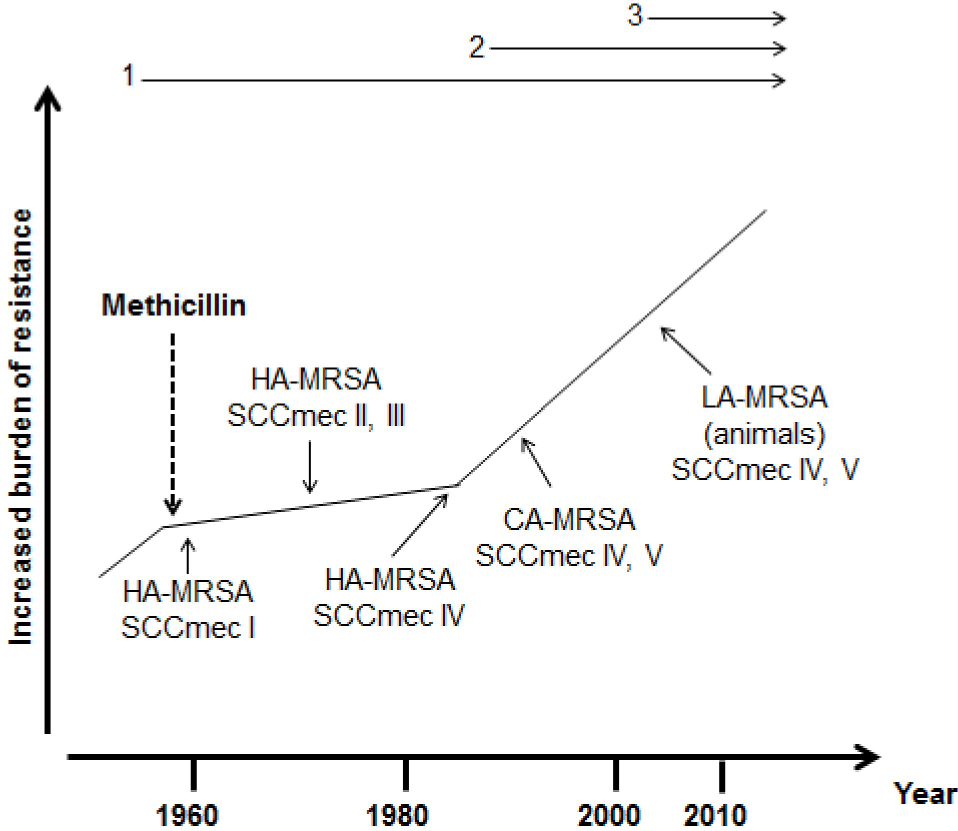

Figure I-3 The description of the waves of antibiotic resistance in *S. aureus* over time. The dotted line indicates the introduction of methicillin. 1) hospital-associated MRSA (HA-MRSA), 2) community-associated MRSA (CA-MRSA) and 3) livestock-associated MRSA (LA-MRSA) (adapted from Chambers and DeLeo, 2009).

2.3 Hospital-associated methicillin-resistant *Staphylococcus aureus*

The intensive use of penicillin resulted in the presence of penicillin-resistant *S. aureus* isolates in the hospitals and community (Jessen et al., 1969; Chambers, 2001). Due to this penicillin-resistance, new antimicrobials were needed. End 1950, a semi-synthetic version of penicillin was produced, named methicillin. Only two years after the first clinical use, MRSA strains emerged in the hospital environment, resulting in the name hospital-associated or HA-MRSA (Jevons et al., 1963).

The evolution of HA-MRSA over time can be divided into two parts (Chambers and DeLeo, 2009). First, the early hospital MRSA strains all belonged to the archaic clones of MRSA. These clones were found in European and US hospitals until the 1970s and none of these strains were found in the rest of the world or in the community. Enright and colleagues (2002) identified the ancestral strain. Multilocus Sequence typing (MLST) classified this strain into clonal complex (CC) 8, which consists of different sequence types (ST) including ST8 (See section 3.3.1.). In CC8, MSSA strains were also present, belonging to ST8. These MSSA strains acquired SCC*mec* type I and evolved to MRSA strains, belonging to ST250. In the 1980s, archaic MRSA isolates had largely disappeared from the European hospital environment, introducing the second part of the evolution. Descendants from the archaic clones (e.g. Iberian or Roman clones) and other successful lineages (e.g. EMRSA-15) had replaced the “original” strains. These new strains carried SCC*mec* types II and III and were found in the hospital environment and other institutional healthcare settings worldwide and are still present today. More recently, SCC*mec* type IV carriage has been described in certain HA-MRSA clones, such as USA800 (CC5-ST5), USA500 (CC8-ST8), EMRSA-15 (CC22-ST22) and CC5-ST200 (Deurenbergh and Stobberingh, 2008; Strandén et al., 2009; Sola et al., 2012). It has been suggested that the same MSSA strain contracted different SCC*mec* cassettes over time (Crisostomo et al., 2001; Enright, 2003; Barada et al., 2007). At present, most HA-MRSA lineages belong to CC5, CC8, CC22, CC30 and CC45.

As mentioned above, HA-MRSA is found worldwide, but the prevalence differs from country to country. Most HA-MRSA strains are multiresistant to antibiotics used in human medicine such as macrolides and aminoglycosides. Some strains are only sensitive to vancomycin, but at present vancomycin resistant isolates have already been detected (Enright, 2003). The mortality rate of invasive MRSA infections is approximately 20% (Stefani et al., 2012). In Europe, the percentage of invasive MRSA isolates ranges from 0.3% in Norway to 54.6% in

Portugal. In countries such as the Netherlands, Norway, Sweden and Denmark, a percentage of ≤1-5% is found (ECDC, 2010). This difference is thought to be maintained by active "Search and Destroy" policies, applied in the hospitals of these countries. The main goal of these policies is to actively search for MRSA-carriers by means of screening certain risk-groups (e.g. in the Netherlands: persons who resided in a foreign hospital or a Dutch hospital with an MRSA outbreak, persons having contact with a MRSA-carrier and occupational contact with pigs and cattle). Until the screening results are known, these persons are treated in isolation. In case of MRSA carriage, the patient remains in isolation and is treated with antibiotics, often together with the health care workers (Wertheim et al., 2004; van Rijen and Kluytmans, 2009; Wassenberg and Bonten, 2010).

2.4 Community-associated methicillin-resistant *Staphylococcus aureus*

Early 1990s, MRSA infections arose in patients without previous healthcare exposure. Phylogenetic studies indicated that these infections were caused by other MRSA lineages than HA-MRSA (Aires de Sousa and de Lencastre, 2003; Huang et al., 2006). Moreover, it is suggested that these lineages evolved separately from the HA-MRSA and acquired the *mec*A gene on a different occasion. A proposed ancestor for community-associated MRSA or CA-MRSA is a descendant of phage type 80/81. This descendant first acquired Panton-Valentine leukocidin (PVL), a bi-component cytotoxin that activates neutrophils and forms pores in these phagocytes, resulting in an exaggerated inflammatory response. In a second step, methicillin-resistance was contracted via SCC*mec* type IV. Subsequently, this strain emerged in the community (Robinson et al., 2005). At present, the CA-MRSA lineages belong to ST1 (US/Canada), ST8 (US/Canada), ST22 (UK), ST30 (Australia), ST59 (Taiwan) and ST80 (Europe). CA-MRSA is more virulent than HA-MRSA. When comparing HA-MRSA and CA-MRSA, differences were observed in the mobile genetic elements (MGE), which may have an impact on transmission, antimicrobial resistance, virulence and severeness of infections (Baba et al., 2002; Lindsay and Holden, 2004; Chambers and DeLeo, 2009; Tong et al., 2011; Moellering, 2012). CA-MRSA isolates carry the SCC*mec* cassettes type IV and V, which are smaller compared to the HA-MRSA cassettes and give the advantage of a higher fitness of the bacterium (Baba et al., 2002). Less additional genes are situated on cassettes type IV and V. As a result, CA-MRSA isolates were only resistant to β-lactam antibiotics and susceptible to antibiotics used in the clinical environment. At present, CA-MRSA strains

appear to become increasingly resistant to other antibiotics, such as erythromycin and fluoroquinolones (Moellering, 2012).

CA-MRSA causes skin and soft tissue infections, but also severe infections such as rapid progressive pneumonia. Risk groups for this type are remote populations, intravenous drug users, men who have sex with men, prison inmates, contact sport teams, military recruits and children. Another important risk factor for the spread of CA-MRSA was intercontinental traveling (Kaiser et al., 2004; Zinderman et al., 2004; Lowy et al., 2007; Chambers and DeLeo, 2009; Giuliani et al., 2010; Lloyd-Smith et al., 2010; Morrison-Rodriguez et al., 2010; Tong et al., 2011; Stefani et al., 2012).

2.5 Livestock-associated methicillin-resistant *Staphylococcus aureus*

From 1970 to 2000, human MRSA strains were sporadically isolated from companion animals, horses and cows (Leonard and Markey, 2008). In 2005, a Dutch and French study reported on MRSA related to pigs. Voss et al. (2005) found upon preoperative screening, a MRSA colonized six-month old girl, who remained colonized after multiple decolonization steps. Testing of the family revealed MRSA carriage in the family members. This family resided on a pig farm. Few months later, MRSA was found in a pig farmer and the son of a veterinarian who worked with pigs. Again, decolonization of the patients failed. Additionally, sampling of the pigs, originating from the pig farm of the six-month old girl and neighbor farms, revealed the presence of MRSA in pigs and persons working closely to pigs (Huijsdens et al., 2006). Armand-Lefevre and colleagues (2005) studied the risk of pig farmers for carrying *S. aureus* in comparison to non-pig farmers. They revealed a higher risk of nasal *S. aureus* carriage in farmers and additionally the carriage of “pig-specific” strains. Independently of each other, both Voss et al. (2005) and Armand Lefevre et al. (2005) described a new MRSA type associated with pigs. When performing Pulsed Field Gel Electrophoresis (PFGE) using the standard *Sma*I restriction protocol, this new MRSA type appeared non-typeable (Voss et al., 2005). MLST typing of the French isolates, revealed the presence of ST398 which was not found in humans before, resulting in the name MRSA ST398. Besides MRSA ST398, other genetic lineages –although at a lesser extent- have been described in livestock animals. In pigs, human lineages were isolated such as CC97 in Spain, CC8-ST8 in Germany, CC1-ST1 in Italy and CC5-USA100 in Canada (Khanna et al., 2008; Gomez-Sanz et al., 2010; Franco et al., 2011; Sunde et al., 2011). The more pig associated lineage, CC9-ST9 has been described in Asia (Cui et al., 2009; Wagenaar et al., 2009; Ho et

al, 2012). Richter and coworkers (2012) reported on CC398 and CC197 isolates in turkeys. In cattle, bovine associated MRSA types were described being CC705 and CC130, often associated with the new *mec*C gene (Garcia-Alvarez et al., 2011; Laurent et al., 2012: Petersen et al., 2013). As in Europe, MRSA ST398 is the most important LA-MRSA clone in livestock animals, the focus of the following section will be on this clone.

2.5.1 Origin of MRSA ST398

Little is known about the origin of MRSA ST398. Guardabassi and colleagues (2007) found ten ST398 strains in a collection of pig *S. aureus* isolates. The occurrence of nine MSSA and one MRSA strain suggested that ST398 MSSA is a commensal bacterium of pigs, which has acquired methicillin-resistance over time. Price and collegues (2012) performed whole-genome analysis on CC398 MSSA and CC398 MRSA isolates from different origins (e.g. human, pig, bovine, turkey and horse). The human MSSA isolates formed the basal clades, indicating that these strains were the most ancestral of the tested isolates. One conclusion of the study was that a MSSA CC398 strain was the ancestor of MRSA ST398 that made a human-to-pig jump. Baquero (2012) indicated that when this jump occurred, the bacterium was not well adapted to its new host and encountered a low fitness. During transmission between the pigs, the bacterium adapted to the pigs by losing human virulence genes and acquiring methicillin and tetracyclin resistance. In livestock farming, a lot of tetracyclin was used as growth promotor and used nowadays for group treatments of the animals. Methicillin resistance was most likely obtained by selective pressure of other antibiotics used in livestock farming such as cephalosporins and others. Heavy metals can also create a selective pressure. Zinc is a metal that is often used in livestock food. Cavaco and colleagues (2010 and 2011) found that zinc and cadmium resistance was linked to methicillin resistance (genetic and phenotypic link). Moodley et al (2011b) evaluated the effect of zinc and tetracyclin on the MRSA ST398 colonization of 2-weeks old piglets. When feed was supplemented with one of both components, the numbers of MRSA ST398 increased in the nasal cavities of the piglets. All these adaptations increased the fitness of MRSA ST398 in pigs (Baquero, 2012). Baquero (2012) also stated that when this adapted MRSA ST398 strain is transmitted to humans again, its fitness is low and subsequently transmission between humans will be difficult. Moreover, during a Dutch study, it was observed that MRSA ST398 is 2.9 times less transmissible than non-ST398 MRSA (Bootsma et al., 2011). In animal MRSA lineages, specific *S. aureus* pathogenicity islands were identified (McCarthy et al., 2012).

2.5.2 Characteristics of MRSA ST398

At present, pig-associated MRSA is found in different animal species and especially livestock animals, resulting in the name change to livestock-associated MRSA (LA-MRSA) or MRSA ST398. LA-MRSA has some specific features that distinguish this type from the other two MRSA types. First, LA-MRSA is non-typeable with Pulsed Field Gel Electrophoresis (PFGE) using *Sma*I restriction. Second, antibiotic resistance for antibiotics used in livestock farming has been observed. Third, in general, LA-MRSA does not carry human virulence genes. Last, more variation was observed in the SCC*mec* carriage: MRSA ST398 mainly carries SCC*mec* type IVa and V although at present others (e.g. SCC*mec* type III, V(5C2&5), IX and X) have been described (Bens et al., 2006; Hallin et al., 2011; Jamrozy et al., 2012; Li et al., 2011; Smith and Pearson, 2011). In general, LA-MRSA is PVL negative, but PVL positive strains have been reported (Osadebe et al., 2012). Some of these features will be further discussed in section 3. LA-MRSA has the ability to acquire additional antibiotic resistance genes (such as *dfr* (trimethoprim) and *erm* (macrolides)) and mobile elements with for example human virulence genes (See section 3.3.6.2.) (Schijffelen et al., 2010; Smith and Pearson, 2011; Kadlec et al., 2012).

2.6 Transmission of Livestock-associated MRSA

When considering animals as a potential MRSA source for the general human population, different transmission routes can be defined. Figure I-4 shows a scheme with potential routes from a farm to the human population which will be further discussed with a focus on MRSA ST398 or, when no data is available, on other (human) MRSA types.

2.6.1 Farm to farm transmission (Figure I-4-route 1)

Three studies indicated that pig trading plays an important role in the dissemination of MRSA ST398. It has been reported that when colonized pigs are sold to farms, the same MRSA ST398 strain as isolated on the supplying farm is frequently detected on these farms. However, some receiving farms had MRSA-positive pigs even though the supplying farm tested MRSA-negative.

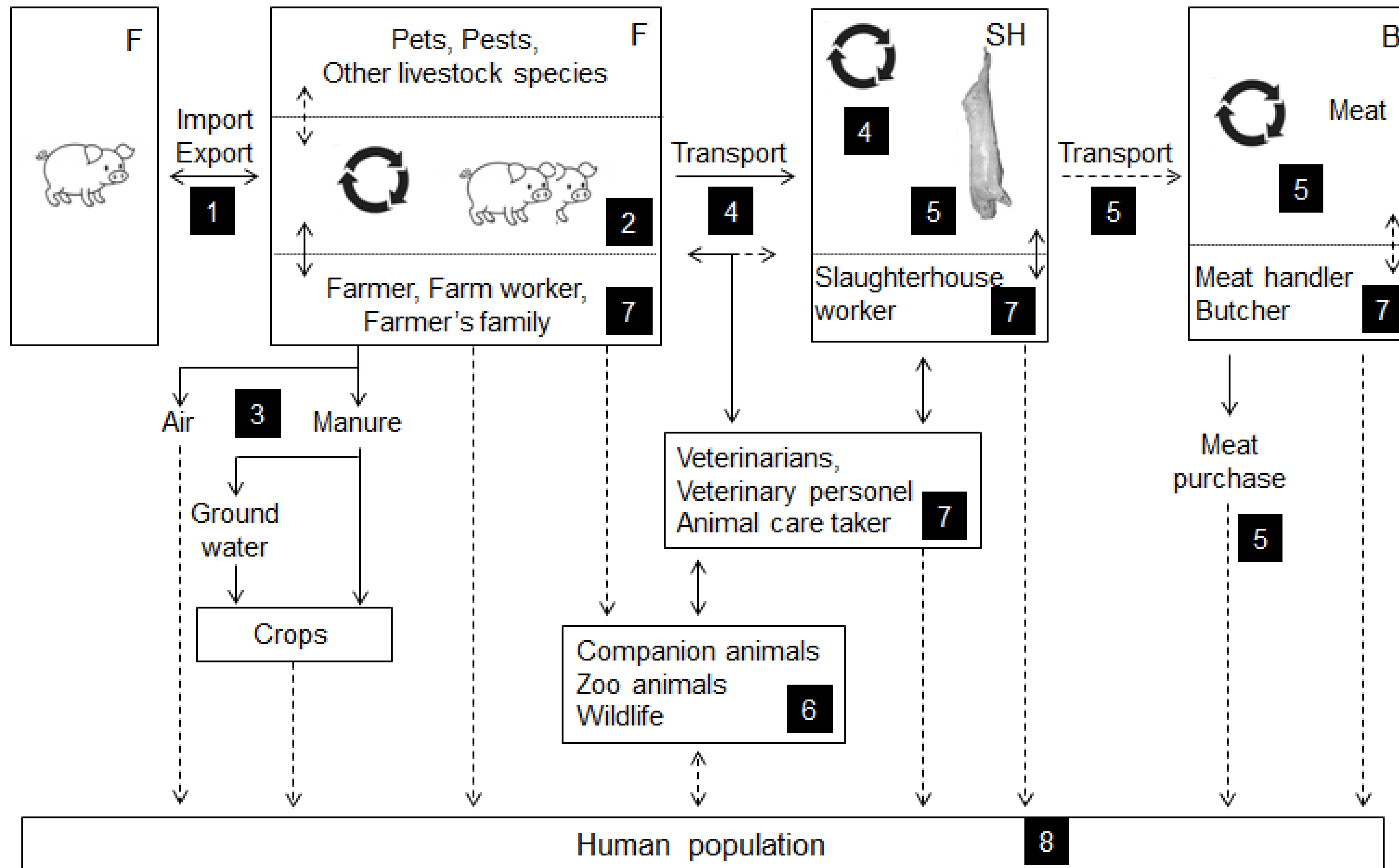

Figure I-4 Overview of possible transmission routes: 1) farm to farm, 2) within a farm/animal group, 3) farm to the human population through the environment, 4) farm to slaughterhouse and within the slaughterhouse, 5) contamination of pig carcasses and pork/pork products, transmission from the slaughterhouse to butcher, within butcheries and transmission via meat purchase, 6) non-livestock animals, 7) persons with higher risk of MRSA colonization and 8) MRSA ST398 in the human population. The dotted line indicates routes on which no reports were published (F: Farm, SH: slaughterhouse, B: Butchery).

This indicates that pig trading is not the only factor in the dissemination of MRSA (Van Duijkeren et al., 2008; Broens et al., 2011a; Espinosa-Gongora et al., 2012a). International pig trade has also been described to facilitate the spread of MRSA ST398 (Wulf and Voss, 2008).

2.6.2 Transmission within a farm (Figure I-4-route 2)

A farm is a dynamic system in which LA-MRSA can be spread around. In this section, the animal groups present at the farm will be addressed: livestock animals (pigs, poultry and cattle), pests and pets. Additionally, reports on transmission within a farm, within an animal group and the role of the environment will be discussed. The role of farmers, farmers' family, farm workers and veterinarians will be described in section 2.6.7.

2.6.2.1 Livestock animals

- Pigs

MRSA ST398 has been found primarily in pigs. Prevalence ranges from 0% in Ireland to 86% in Spain (Table I-2). Risk factors for increased pig MRSA carriage are: the use of antibiotics (group treatments), larger herds and the presence of fattening pigs (Alt et al., 2011; Broens et al., 2011a). In most cases, pigs are asymptomatic carriers of this bacterium (Meemken et al., 2008; Szabó et al., 2012). However, there have been a limited number of reports on infections in pigs such as skin infections, exudative epidermitis, urogenital tract infections, uterus and mammary gland infections (Van Duijkeren et al., 2007; Schwarz et al., 2008; Meemken et al., 2010; Pomba et al., 2010).

- Poultry

MRSA ST398 is usually less frequently found in poultry. In Belgium, Nemati and co-workers (2008) described MRSA ST398 for the first time in poultry. Moreover, a Belgian prevalence of 0.0% in layers and 10.7% in broilers (*spa* type t1456) was found (Persoons et al., 2009). Pletinckx et al. (2011 and 2013a) found a prevalence of 7.2% and 18.7% in broilers. In two Dutch studies, low prevalences were observed (4.4%-6.9%) and isolates belonged to ST398 and ST9 (Mulders et al., 2010; Geenen et al., 2012). Nevertheless, in Germany, a prevalence of 71.5% was observed in turkeys (Richter et al., 2012). A more recent German study reported a prevalence of 62-80% in turkeys and of 50.0%-54.0% in poultry on farms with a previous known positive MRSA status (Friese et al., 2013a). Vandendriessche and coworkers (2013) found MRSA on one out of twenty sampled poultry farms.

Table I-2 European and American/Canadian MRSA prevalence studies in pigs from 2004 onwards. Sampling occurred at the slaughterhouses or the farms. Only studies where nasal swabs were taken are shown. For ST398, only the *spa* type is mentioned. For non-ST398, the ST and *spa* type is given.

MRSA prevalence % (total number of samples)[a,b]	Number of positive farms/total [b,c]	Isolate characteristics[b]	Country, year[b]	Reference
Slaughterhouse				
10.0 (n=100)		t034, t1793	Denmark, 2005	Bagcigil et al., 2007
41.5 (n=504)	44/54	t011, t108, t1254, t1255, t567, t943, t034	The Netherlands, 2005	de Neeling et al., 2007
0.0 (n=400)			Ireland, 2007	Horgan et al., 2011
NA (Pooled)	45/118	t011, t034, t108, t2510, t4838, t2922, t899 ST9-t4794 / ST97-t4795 / ST1-t127 / ST1476-t1730	Italy, 2008	Battisti et al., 2010
64.7 (n=133)	56/79	t034, t108, t1606, t2346	Germany, 2008-2009	Beneke et al., 2011
35.0 (n=106)			Spain, 2008-2009	Gomez-Sanz et al., 2010
21.0 (n=53)	Finishing pigs	t011, t108 CC97-ST1379-t3992		
49.1 (n=53)	Suckling piglets	t011, t1197, t2346		
1.3 (n=800)		t034	Switzerland, 2009	Huber et al., 2010
12.8 (n=789)		t011, t034, t1451; t2876, t2974, t1333 CC1-t187	Denmark, 2009	Agersø et al., 2012
85.7 (n=300)		NA	Spain, 2009-2010	Morcillo et al., 2012
2009: 2.0 (n=405)		t011, t034, t1451 ST1-t2279 / ST49-t208	Switzerland, 2009-2010	Overesch et al., 2011
2010: 5.9 (n=392)		t011, t034 ST49-t208		
0.0 (n=304)	0/54		Switzerland, NA	Riesen and Perreten, 2009
60.0 (n=1026)		t011, t034, t108, t145, t571, t1250, t1255, t1451, t1580, t1928, t1985, t2011, t2346, t2576, t2970, NT	Germany, NA	Tenhagen et al., 2009
11.2 (n=240)	5/10	t539, t5883 / ST5-t002	US, NA	Molla et al., 2012
30.8 (n=26)	1/1	t034 / ST-8-t064	Canada, NA	Hawken et al., 2013

MRSA prevalence % (total number of samples)[a,b]	Number of positive farms/total [b,c]	Isolate characteristics[b]	Country, year[b]	Reference
Farm				
46.0 (n=50)	4/5	t034	Denmark, 2004-2007	Lewis et al., 2008
11.3 (n=310)	7/31	t011, t899, t567, t108, t1939	The Netherlands, 2006	Van Duijkeren et al., 2008
44.2 (n=1500)	34/50		Belgium, 2007	Crombé et al., 2012a
	Closed farms: 19/34	t011		
	Fattening farms: 15/16	t034, t567		
65.4 (n=412)	2/2	NT with PFGE *Sma*I	Belgium, 2007	Dewaele et al., 2011
75.8 (n=223)	Farm A			
55.0 (n=189)	Farm B			
NA (Pooled)	28/40	t011, t108, t034, t1451, t2510	Germany, 2007	Köck et al., 2009
12.5 (n=678)	62/347	NA	Germany, 2007	Meemken et al., 2008
NA	28/50	t011, t108, t567, t899, t2330	The Netherlands, 2007	van den Broek et al., 2008
53.6 (Pooled) 63.8 (Pooled)	Finishing farms (22/31) Breeding farms (115/171)	t011, t108, t1457, t899, t567, t1184, t571, t2330, t1456, t2346, t034, t588, t3479, t943, t1451, t2011, t4119 ST1-t127 / ST5-t002	The Netherlands, 2007-2008	Broens et al., 2011a
NA	2/2	t011 ST30-t021	Portugal, 2008	Pomba et al., 2009
low	NA	t108	Finland, 2008	Salmenlinna et al., 2012
1.3 (n=157)	State fair (1/2)	t3075 ST2136-t337	US, 2008-2009	Dressler et al., 2012
NA	16/17	t011, t034; t2576	Germany, 2008-2009	Fessler et al., 2010
4.6 (n=1085)	9/43	t034, t571 ST5: t002 / ST NA-t337 / ST NA, t3446 / NT	US, 2008-2010	Smith et al., 2013
74.0 (n=311)	4/4	t011, t034	Denmark, 2009	Espinosa-Gongora et al., 2012b
89.5 (n=209)	4/4		Belgium, 2009-2010	Pletinckx et al., 2009
86.2 (n=29)	Farm A	t011		
70.0 (n=60)	Farm B	t011		
100.0 (n=60)	Farm C	t011, t567		
100.0 (n=60)	Farm D	t567		

MRSA prevalence % (total number)[a,b]	Number of positive farms/total [b,c]	Isolate characteristics[b]	Country, year[b]	Reference
63.0 (n=1929)	6/6		Belgium, 2009	Pletinckx et al., 2013a
39.8 (n=324)	Farm A	t034, t567, t5943		
53.2 (n=310)	Farm B	t011, t034		
82.8 (n=325)	Farm C	t011		
83.7 (n=320)	Farm D	t011		
54.8 (n=325)	Farm E	t011, t034		
63.4 (n=325)	Farm F	t011		
86.3 (n=972)	3/3		Belgium, 2009-2010	Pletinckx et al., 2011
83.7 (n=325)	Farm A	t011		
82.8 (n=325)	Farm B	t011		
92.5 (n=322)	Farm C	t011, t1451		
3.0 (n=263)	5/35	t034 ST8-t008 / ST39-t007	US, 2009-2010	Osadebe et al., 2012
17.4 (n=194)	12/40	t034 ST5: t002, t548	US, 2010	Frana et al., 2013
82.0 (n=200)	10/10	t011, t108, t2970, t2330	Belgium, 2009-2011	Vandendriessche et al., 2013
0.0 (n=60)	NA		US, 2010-2011	Buyukcangaz et al., 2013
2.5 (n=160)	NA/8	t011	Lithuania, 2011	Ruzauskas et al., 2013
0.0 (n=97)	NA		UK, NA	Nunan and Young, 2007
24.9 (n=285)	9/20	t034 ST5-NA	Canada, NA	Khanna et al., 2008
49.2 (n=299)	2/2	NA	US, NA	Smith et al., 2009
7.0 (n=86)	1/1	ST45-t015	Serbia, NA	Velebit et al., 2010
59.5 (n=259)	2/2		US, NA	Larson et al., 2011
14.0 (n=50)	Farm A	ST NA-t1576		
70.3 (n=209)	Farm B	t034		
4.6 (n=460)	5/46	t011, t034, t4652 ST5: t002, t5518, t067 ST8-t064	Canada, NA	Weese et al., 2011
0.0 (n=178)	0/25[d]		Germany, NA	Cuny et al., 2012
NA (Pooled)	23/27	t011, t108, t034, t1255, t1451	Germany, NA	Friese et al., 2012

MRSA prevalence % (total number)[a,b]	Number of positive farms/total [b,c]	Isolate characteristics[b]	Country, year[b]	Reference
3.0 (n=240)	3/10	new ST5: t002, t5883 ST9-t1435	US, NA	Molla et al., 2012

[a] NA (Pooled): no animal prevalence is available, because pooling of the samples occurred.
[b] NA: Not available, NT: Non-typeable
[c] When available, the number of positive farms/total is indicated. For some studies, additional information is given.
[d] Alternative farms

- Cattle

No MRSA was isolated from cattle in a Danish, American and Canadian study (Lewis et al., 2008; Peterson et al., 2012; Weese et al., 2012; Buyukcangaz et al., 2013). In Swiss cattle and calves at slaughter age, the MRSA prevalence was 0.3% (ST1) and 3.0% (ST398), respectively (Huber et al., 2010). In French and Dutch veal calves, a prevalence of 6.5% and 28.0%, respectively, was observed (Graveland et al., 2010; Haenni et al., 2011). Two German studies reported a MRSA ST398 prevalence in cows of 46.6% and 12.5%, respectively, and in calves of 57.1% and 18.2%, respectively (Spohr et al., 2010; Fessler et al., 2012). A recent Belgian study described a prevalence of 64.0%, 1.0% and 5.0% in veal calves, dairy cattle and beef cattle, respectively (Vandendriessche et al., 2013). On two Belgian farms, in dairy cows, a prevalence of 7.1% and 86.7% was found, whereas in calves a prevalence of 12.5% and 13.3%, respectively (Pletinckx et al., 2013a). Risk factors for increased MRSA isolation are: group treatment with antimicrobials, age and rodent control (Van Duijkeren et al., 2008; Graveland et al., 2010; Bos et al., 2012). One of the infections that LA-MRSA is thought to cause in cattle, is mastitis. In most reports, the sampling locations and/or strain types were not described, which makes the estimation of LA-MRSA prevalence and contribution in mastitis very difficult. During a Belgian study, a prevalence of 9.3% was reported (Vanderhaeghen et al., 2010b). In the Netherlands, analysis of 38 000 milk samples, obtained from cows with (sub)clinical mastitis, resulted in the isolation of 14 MRSA ST398 strains (1 strain per herd) (Tavakol et al., 2012). A recent Belgian study identified 18 MRSA ST398 strains amongst a *S. aureus* collection isolated from milk of cows suffering from mastitis (Bardiau et al., 2013).

2.6.2.2 Pests

Black and brown rats have been shown to carry MRSA ST398 on Belgian and Dutch pig and veal farms. The authors indicated that rats may play a role in the spread and persistence of MRSA on farms (van de Giessen et al., 2009; Pletinckx et al., 2013a).

2.6.2.3 Stable pets

Pletinckx and colleagues (2010 and 2013a) found MRSA ST398 in Belgian dogs and cats, which resided in the pig stables or came into contact with the pigs.

2.6.2.4 MRSA ST398 within a farm

On farrow and farrow-to-finish farms, piglets are born on site. At present, few studies have been performed on the colonization status of piglets from birth onwards (Nathaus et al., 2010; Weese et al., 2010a; Broens et al., 2012a; Hawken et al., 2013). When comparing the results of these studies, different colonization trends were observed (Figure I-5). During various studies, an age dependent colonization was observed. When the animals were older (sows, slaughter age), a decrease in MRSA isolation was observed (Denis et al., 2006; Broens et al., 2011a; Dewaele et al., 2011; Pletinckx et al., 2013a).

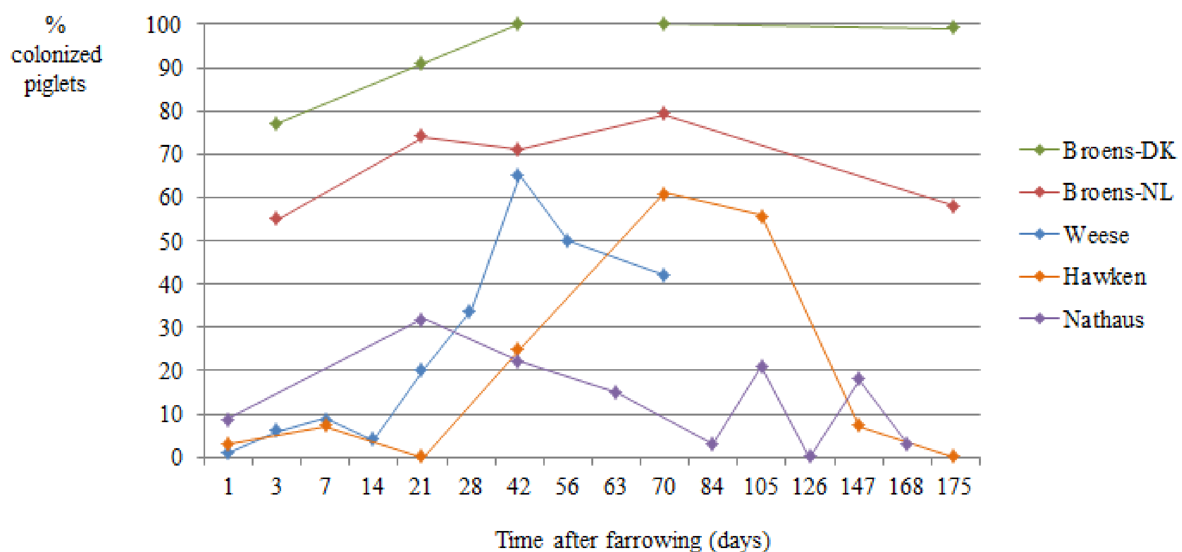

Figure I-5 Colonization profiles of piglets as observed in four studies over time (Weese et al., 2010a; Nathaus et al., 2010; Broens et al., 2012a and Hawken et al., 2013). Broens and colleagues (2012a) determined the piglet prevalences of Dutch (NL) and Danish farms (DK). When more than one group of piglets was studied, the average prevalences per sampling event are shown (Nathaus et al., 2010; Broens et al., 2012a).

At present, only one study has conducted on the presence of MRSA ST398 in different animal species (livestock, pets and pests) and humans present within one farm. The authors identified the same MRSA strain in the animals, the environment and the humans, suggesting a possible MRSA transfer within the farm (Pletinckx et al., 2013a).

2.6.2.5 Transmission within an animal group

To study the MRSA spread within a group of piglets, few colonization experiments have been described (Moodley et al., 2011a and 2012; Szabó et al., 2012; Broens et al., 2012a and b; Crombé et al., 2012b; Gibbons et al., 2013). In general, exposure of MRSA-negative piglets to MRSA positive piglets resulted in fast colonization of the negative ones (within 1 to 7

days). The reproduction ratios were larger than one, which is an indication that one colonized animal is able to infect more than one non-colonized animal during the entire infectious period (Broens et al., 2012b; Crombé et al., 2012b). Moreover, colonization with a MRSA ST398 strain resulted in a more persistent colonization in comparison to ST8 and ST9 strains (Szabó et al., 2012). Remarkably, when the piglets were inoculated with MSSA before MRSA, no persistent colonization was observed (Broens et al., 2012b). Inoculation of the skin (sacral region) of gnotobiotic piglets resulted in MRSA-isolation from the nares and skin behind the ears after 2 days (Giotis et al., 2012). Besides from the nares and skin, MRSA ST398 was also isolated from the rectum (Moodley et al., 2011a and 2012; Szabó et al., 2012; Broens et al., 2012b; Crombé et al., 2012b).

It appeared that a high inoculation dose of 10^8 colony forming units (cfu) was needed to maintain a persistent nasal colonization of pigs (Moodley et al., 2011a; Szabó et al., 2012; Broens et al., 2012b; Crombé et al., 2012b). However, the study of Jouy and colleagues (2012) indicated that an inoculation dose of 10^4 cfu was sufficient to induce transient nares contamination and horizontal transmission of MRSA, but not persistent MRSA carriership.

2.6.2.6 Role of the stable/farm environment

In stables, where pigs are housed, MRSA ST398 can be found in the environment. MRSA ST398 was found in the air, dust, on the walls, on the floor and horizontal surfaces. The same MRSA strain as seen in the animals could be isolated from the environment (van den Broek et al., 2008; EFSA, 2009; Spohr et al., 2010; Dewaele et al., 2011; Espinosa-Gongora et al., 2012b; Friese et al., 2012; Richter et al., 2012; Schulz et al., 2012). MRSA could also be retrieved from animal feed, manure and drinking water (Friese et al, 2012). Environmental contamination was also observed in poultry and turkeys (Geenen et al., 2012; Richter et al., 2012). Friese and collegues (2012) reported a positive correlation between the proportion of MRSA positive pigs and the number of positive air samples. Moreover, long persistence of LA-MRSA on surfaces is expected since HA-MRSA and CA-MRSA can survive up to 4 months on surfaces (Desai et al., 2011; Petti et al., 2012). In all colonization experiments, described in the previous section 2.6.2.5., MRSA was detected in the environment (wall, floor and/or air) once the animals were inoculated and in the report of Moodley et al. (2011a) once the MRSA-positive animals were brought in the stables. Gibbons and co-workers (2013) described environmental transmission of a CC5-t002 MRSA strain to MRSA-negative pigs.

2.6.3 Transmission from the farm environment to the human population (Figure I-4-route 3)

In 2006, Gibbs et al. and Green et al. reported on multidrug resistant *S. aureus* strains (<125 cfu/m³) up to 150m from a pig farm. During a more recent study, LA-MRSA was isolated 50 and 150m downwind from pig barns, with a concentration of 11-14 cfu/m³ (Schulz et al., 2012). All authors suggested a possible transmission route to the general human population when residing near pig farms.

Besides from the air, Schulz and coworkers (2012) also isolated LA-MRSA from the soil surfaces at distances of 50, 150 and 300m from a pig barn. MRSA ST398 was also isolated from manure (Nunan and Young, 2007; Friese et al., 2013b). Nunan and Young (2007) suggested that when manure is used on the fields, the seeded crops may become contaminated. Moreover, ground water might get contaminated. When this water is used to irrigate crops, such as lettuce, the human population might get exposed to MRSA.

2.6.4 Transmission from the farm to the slaughterhouse and within the slaughterhouse (Figure I-4-route 4)

Broens and colleagues (2011b) studied the nasal MRSA status of pigs, originating from a non-colonized farm, during loading at the farm, on arrival at the slaughterhouse and after stunning. The prevalence was 0% at loading and 10% upon arrival at the slaughterhouse. However, when the animals were transported in a lorry where no MRSA was detected, none of the animals were MRSA colonized. The MRSA prevalence of pigs was 60% at stunning after 1 to 11 hours in the lairage, which is an indication of MRSA transmission within the lairage. Moreover, it has been reported in pig and broiler slaughterhouses that the environmental contamination of MRSA increased along the slaughterline during the day (Mulders et al., 2010; Van Cleef et al., 2010a; Gilbert et al., 2012).

2.6.5 MRSA on pig carcasses, pork/pork products and possible transmission to the human population (Figure I-4-route 5)

To date, the presence of MRSA on pig carcasses and on pork has not been abundantly studied. Since the use of different methodologies makes comparison between the reports difficult, an overview of the literature is shown in Table I-3. In general, few MRSA and MRSA ST398 were found in those studies and most MRSA strains belonged to human clones.

Table I-3 Overview of the European and American/Canadian literature regarding the MRSA isolation from pig carcasses and pork. For ST398 isolates, only the *spa* type is shown, whereas for non-ST398 isolate, the ST and *spa* type is given. All studies, except Kelman et al. (2011), determined the prevalence after sample enrichment.

Sample	MRSA prevalence % (n=total number)	Isolate characteristics[a]	Country, Year[a]	Reference
Pig carcass	0.0 (n=200)		Switzerland, 2006	Spescha et al., 2006, Nitzsche et al., 2007
Pork	3.1 (n=64)	t108 ST8-t024	The Netherlands, 2006	Van Loo et al., 2007
Raw meat	0.0 (n=15)		Portugal, 2006-2008	Pereira et al., 2009
Dutch pork/sausage	0.0 (n=28)		UK, 2007	Nunan and Young, 2007
Pork	0.0 (n=12)		US, 2007	Chan et al., 2008
Pork, beef, poultry	0.0 (n=176)		Italy	Pesavento et al., 2007
Pork	10.7 (n=309)	t011, t108, t034, t567, t899, t1451 ST45/47-t026	The Netherlands, 2007-2008	de Boer et al., 2009
Pork Wild boar	1.8 (n=55) 25.0 (n=4)	t011 ST217-t032	Spain, 2007-2009	Lozano et al., 2009
Pork	2.0 (n=50)	NA	UK, NA	Fielder, 2007
Pork	40.0 (n=10)	t011	The Netherlands, 2008	de Jonge et al., 2010
Ground pork	0.3 (n=300)	ST5-USA100	US, 2008	Kelman et al., 2011
Pork	5.6 (n=90)	ST8-t008 ST5-t002	US, 2008	Pu et al., 2009
Retail pork	7.7 (n=402)	t034 ST8: t064, t008 ST5: t002, t045	Canada, 2008	Weese et al., 2010b
Pork chops	7.8 (n=296)			
Ground pork	7.4 (n=94)			
Pork shoulder	8.3 (n=12)			
Pork	9.6 (n=230)	ST5-t242	Canada, 2008-2009	Weese et al., 2010c
Ground pork	6.3 (n=127)			
Pork chops	13.6 (n=103)			

Sample	MRSA prevalence % (n=total number)	Isolate characteristics[a]	Country, Year[a]	Reference
Pork	4.2 (n=144)	t011, t034, t2510	Germany, 2008-2009	Beneke et al., 2011
Shoulders	2.1 (n=48)			
Bellies	4.2 (n=48)			
Backs	6.3 (n=48)			
Final products (goulash, minced meat, sausage)	2.8 (n=71)			
Minced pork/beef	0.0 (n=160)		Switzerland, 2009	Huber et al., 2010
Pork (Chops and ground pork)	3.6 (n=55)	t034 ST8-t008	US, 2009	Hanson et al., 2011
Danish pork	4.6 (n=153)	t034	Denmark, 2009	Agersø et al., 2012
Imported pork	7.5 (n=173)	t011, t108		
Germany	2.1 (n=142)			
The Netherlands	18.8 (n=16)			
France	100.0 (n=1)			
Poland	100.0 (n=1)			
Other EU countries	41.7 (n=5)			
Third world countries	0.0 (n=1)			
Pork overall	6.6 (n=395)	t011, t034, t094, t273, t803, t2922 ST5-t002 / ST8-t008 / ST2007-t8413 / ST26-t078	US, 2010	O'Brien et al., 2012
Riblets	0.0 (n=12)			
Blade steak	0.0 (n=9)			
Cube steak	0.0 (n=2)			
Pork loin	0.0 (n=13)			
Pork chops	5.0 (n=141)			
Pork sausage	5.4 (n=56)			
Ground pork	8.3 (n=72)			
Pork ribs	8.5 (n=59)			
Pork roast	8.0 (n=25)			
Pork cutlet	50.0 (n=6)			
Pork	3.8 (n=26)	ST5-NA	US, NA	Waters et al., 2011
Pig carcass	2.0 (n=235)	t337	US, NA	Molla et al., 2012
Pork	4.0 (n=135)	ST5-t002 / ST39-t007 / ST72-t049 / ST9-t337		

Sample	MRSA prevalence % (n=total number)	Isolate characteristics[a]	Country, Year[a]	Reference
Pork	3.0 (n=100)	ST5-t002 / ST9-t337 / ST30-t012	US, 2009	Jackson et al., 2013
Pork chops	6.5 (n=31)			
Ground pork	0.0 (n=14)			
Pork ears	0.0 (n=6)			
Pork feet	0.0 (n=13)			
Pork tails	0.0 (n=4)			
Pork ribs	7.1 (n=14)			
Others (bones and organs)	0.0 (n=12)			
Pork	7.0 (n= 71)	ST398 / ST5	US, 2010-2011	Buyukcangaz et al., 2013
Pig carcass	1.3 (n=78)	new	Canada, NA	Hawken et al., 2013
Bleeding area	3.8 (n=26)			
Preevisceration area	0.0 (n=26)			
Postevisceration area	0.0 (n=26)			

[a] NA: Not available

Only three studies determined the colony forming units of MRSA present on meat. Two Dutch studies estimated the cfu/g meat (between 0.06 and more than 10 cfu/g) after sample enrichment (de Boer et al., 2009; de Jonge et al., 2010). In a Canadian study the colony counts were <100 cfu/g in the majority of pork samples. On three pork samples, 110, 340 and 3590 cfu/g were observed (Weese et al., 2010b).

At present, neither the spread of MRSA from the slaughterhouse to the butcheries nor transmission within butcheries has been investigated. In the US, the same MRSA strain was isolated from the animals as well as the carcasses and meat samples (Molla et al., 2012). Furthermore, no data is available about the transmission of MRSA ST398 from meat to humans. It is not known whether this bacterium spreads easily in a non-farm/non-livestock environment. Food poisoning due to MRSA ST398 is not expected since this strain does not carry the toxin-producing genes (Hallin et al., 2011; Jamrozy et al., 2012). Moreover, *S. aureus* and the three MRSA types are killed upon cooking (Nunan and Young, 2007).

2.6.6 Non-livestock animals (Figure I-4-route 6)

Animals play an important role in the dissemination of MRSA throughout the population. Besides livestock animals (such as pigs, poultry and cattle), companion animals, zoo animals and wildlife animals might carry MRSA.

2.6.6.1 Companion animals

Horses have been described as MRSA carriers (prevalence between 0-11%). Horses may carry i) human lineages, ii) horse specific lineages such as MRSA-5, a CC8 clone or iii) MRSA ST398 (Weese et al., 2005a and b; Cuny et al., 2006; Tokateloff et al., 2009; Van den Eede et al., 2009 Abbott et al., 2010; Weese and Van Duijkeren, 2010; Fessler et al., 2012; Van den Eede et al., 2013a and b). Transmission of MRSA (ST398) was also described between horse owners and their horses (van Duijkeren et al., 2011; Van den Eede et al., 2013a).

Low MRSA prevalences have been described in cats (0.0%-4.0%) and dogs (0.0-9.0%). In most cases, these pets carry human MRSA strains, which may be associated with household members. Moreover, human to pet transmission and *vice versa* has been described (Van Duijkeren et al., 2004; Strommenger et al., 2006a; Weese et al., 2006a; Weese and Van Duijkeren, 2010; Coughlan et al., 2010; Loeffler and Lloyd, 2010; Vanderhaeghen et al.,

2010a) At present, MRSA ST398 is rarely found in household pets. Most often, pets associated with farms, livestock veterinarians or the countryside appear to carry MRSA ST398 (Witte et al., 2007; Nienhoff et al., 2009; Fessler et al., 2012; Haenni et al., 2012); Pletinckx et al., 2013a). In the household of a pig veterinarian, the dog was found to carry the same MRSA ST398 strain (t034) as the owner (Nienhoff et al., 2009).

MRSA has also been described in other animal species such as goats, sheep, rabbits, guinea pigs, turtles, parrots, seals and bats. The strain origin is not always mentioned but in most cases human sequence types were observed (O'Mahony et al., 2005; Briscoe et al., 2008; Walther et al., 2009; Pletinckx et al., 2011; Wieler et al., 2011; Fessler et al., 2012; Paterson et al., 2012; Loncaric et al., 2013a and 2013b).

2.6.6.2 Zoo animals and wildlife animals

Until now, MRSA was sporadically found in zoo animals and wildlife animals. In most cases, human strains were isolated (Faires et al., 2009; Janssen et al., 2009; Schaefer et al., 2009; Paterson et al., 2012; Wardyn et al., 2012; Hower et al., 2013). A recent report describes the presence of MRSA isolates carrying the *mec*C gene originating from hares, an otter and a hedgehog (Loncaric et al., 2013c). In the Copenhagen and Antwerp Zoo, no MRSA was detected (Espinosa-Gongora et al., 2012c; Vercammen et al., 2012).

2.6.7 Risk occupations (Figure I-4-route 7)

At present, low carriage percentages (0.2-9.0%) of MRSA ST398 have been reported in the general human population (Cuny et al., 2009; Van Cleef et al., 2010a; Lozano et al., 2011a). Some occupations represent a high risk for contracting MRSA ST398 through direct contact with the colonized animals. Such risk groups are: i) farmers, farmers' family members and farm co-workers; ii) slaughterhouse workers and meat handlers and iii) veterinarians, veterinary personnel and animal care takers.

2.6.7.1 Farmers, farmers' family members and farm co-workers

In European studies, the MRSA ST398 prevalence of pig farmers and co-workers situates between 0.0% in Switzerland, a low MRSA prevalence country, to 56.0% in Belgium, a high MRSA prevalence country (Huber et al., 2010; Vandendriessche et al., 2013). A prevalence of 20.0% was observed in a Canadian study and in an American study the prevalence was

between 20.9% and 45.0% (Khanna et al., 2008; Smith et al., 2009; Osadebe et al., 2012; Smith et al., 2013).

There is a known occupational risk when working with livestock on daily basis. The more hours spent per week in the stables and the more MRSA positive animals present, the more the risk of being an MRSA carrier increases (Meemken et al., 2008; van den Broek et al., 2008; van Rijen et al., 2008; Wulf et al., 2008; Denis et al., 2009; Graveland et al., 2010; Garcia-Graells et al., 2012; Geenen et al., 2012; Vandendriessche et al., 2013). At present, it is not clear whether a farmer is a long-time carrier or recolonization of the farmer occurs. Köck and coworkers (2012) reported that an absence of two weeks was not sufficient to decolonize all farmers. Moreover, farmers that tested negative after the absence were recolonized after two to three days. This fast recolonization was also observed by Lozano et al. (2010). Nevertheless, people having short-term contact with pigs lose their nasal MRSA colonization within 24 hours (van den Broek et al., 2008; Graveland et al., 2010; Van Cleef et al., 2011). The most important risk factor for human MRSA carriage on broiler farms appears to be working/living on MRSA positive farms (Geenen et al., 2012). On cattle farms, it is the number of MRSA positive animals (Graveland et al., 2011).

The farmers' family members are also at risk for contracting MRSA ST398, but lower prevalences were seen. It has been suggested that this is due to infrequent human-to-human transmission. Higher prevalences were reported in family members with (minimal) pig exposure (12.0%) than in family members without pig exposure (1.8%). In Germany, only 4.3% of non-exposed family members were carrying MRSA (Voss et al., 2005; van Rijen et al., 2008; van den Broek et al., 2008; Cuny et al., 2009; Graveland et al., 2011; Baquero, 2012). Note that the first description of MRSA ST398 was in a family member (see section 2.5., Voss et al., 2005).

Sometimes, human MRSA types were isolated from farmers, family members and co-workers, but in most cases, they carry the same MRSA ST398 type as isolated from the livestock animals, such as pigs and cattle, which was also observed in poultry and turkey farmers (Armand-Lefevre et al., 2005; Juhasz-Kaszanyitzky et al., 2007; Khanna et al., 2008; Denis et al., 2009; Dewaele et al., 2011; Geenen et al., 2012; Richter et al., 2012).

2.6.7.2 Slaughterhouse workers and meat handlers

A second category of humans, coming into contact with livestock animals, are slaughterhouse workers. No MRSA was detected in slaughterhouse workers in Switzerland, a low MRSA prevalence country (Huber et al., 2010). In The Netherlands, where high MRSA ST398 rates are observed, an overall nasal prevalence of 5.6% in slaughterhouse workers was reported. MRSA was mainly retrieved from persons having contact with living animals. Additional MRSA carriers were found amongst the livestock transport workers, official veterinarians and people working in the dirty area of the pig slaughterhouses (Van Cleef et al., 2010b). The previous study indicated that working with live pigs was a risk factor for retrieving MRSA (Van Cleef et al., 2010b). Moreover, a more recent study indicated that working in the lairage and scalding and dehairing area were also major risk factors for retrieving MRSA as a slaughterhouse worker (Gilbert et al., 2012). Due to the low MRSA prevalences seen in the previous studies and the study of Morcillo et al. (2012), it is assumed that the carrier risk of slaughterhouse workers is lower in comparison to livestock farmers.

Meat handlers come in contact with pig carcasses. In the pig slaughterhouse, no MRSA was detected in people working with dead animals (clean area worker, carcass cooling and cutting plant worker, green offal worker, meat hygiene inspector, quality assurance). In addition, no MRSA was isolated from persons working in the cold meat processing industry and in institutional kitchens (de Jonge et al., 2010; Van Cleef et al., 2010b). In Hong Kong, 5.6% of the butchers in the wet markets were colonized with MRSA (ST9) (Boost et al., 2012).

2.6.7.3 Veterinarians, veterinary personnel and animal care takers

Veterinarians come in contact with different animals. In Europe, MRSA prevalences ranging from 0.48% in Denmark to 57.0% in France were observed. In the US and Canada, the prevalence ranged from 6.5% to 10.1%. During Swiss, Danish, Polish, Dutch and Belgian studies, MRSA ST398 was detected (Armand-Lefevre et al., 2005; Loeffler et al., 2005; Weese et al., 2005b; Hanselman et al., 2006; Wulf et al., 2006; Wulf et al., 2007; Anderson et al., 2008; Moodley et al., 2008; Heller et al., 2009; Marszalek et al., 2009; Zemlickova et al., 2009; Huber et al., 2010; Ishihara et al., 2010; Garcia-Graells et al., 2012; Jordan et al., 2011; Paul et al., 2011; Verkade et al., 2013). Pig veterinarians have been shown to carry MRSA ST398 in their nares (Nienhoff et al., 2009). In addition, during the Belgian study of Garcia-Graells and coworkers (2012), working with livestock was considered a significant risk factor

for LA-MRSA carriage. Moreover, veterinarians working with pigs were at higher risk than those working with cattle. The highest risk was associated with exposure to live pigs. Remarkably, no association with MRSA carriership was found when veterinarians came in contact with other farm animals such as sheep, goats, poultry, horses and companion animals (Garcia-Graells et al., 2012).

In addition, veterinary personnel and (zoo) animal caretakers encounter different healthy and sick animals. MRSA carriage of these persons and transmission from animals to these persons has been described before (Baptiste et al., 2005; Loeffler et al., 2005; Weese et al., 2005a; Weese et al., 2005b ; Weese et al., 2006a; Weese et al., 2006b; Janssen et al., 2009; Walther et al., 2009; Van Duijkeren et al., 2010).

2.6.7.4 MRSA ST398 infections

Until approximately 2003, MRSA ST398 was not found in MRSA collections of hospitals worldwide. From then on, an increase in MRSA ST398 was observed which is an indication that this MRSA type is recently emerging in the human population (van Loo et al., 2007; Witte et al., 2007; van Rijen et al., 2008; Wulf et al., 2008; Grisold et al., 2010; Meemken et al., 2010; Wulf et al., 2011; Haenen et al., 2009). In 2005, Voss and colleagues reported that the prevalence rate in farmers was more than 760 times greater than in the general human population. In 2006, a new risk group (persons in contact with livestock) was added to the hospital screening programs in The Netherlands. Fifty-two tetracyclin resistant MRSA strains, originating from a collection of MRSA strains obtained during a one-year period in a Spanish hospital, revealed that 67.3% belonged to CC398 (ST398 and ST2077). However, this hospital was located in a high density pig farming region, which might explain the high recovery of CC398 (Lozano et al., 2011a). In a recent Belgian study, the MRSA admissions between 2006 and 2009 were studied. This study revealed that the proportion of LA-MRSA was below 2.0% (Vandendriessche et al., 2012). In a recent German study, LA-MRSA accounted for 18.6% of all human isolates from 39 hospitals (Köck et al., 2013).

MRSA ST398 infections in farmers or humans with animal contact have been described, such as skin lesions, abscesses, destructive otomastoiditis, skin and soft-tissue infections, bronchitis, chronic obstructive pulmonary disease, folliculitis, wound infections, otitis and blood infections (Witte et al., 2007; Van Belkum et al., 2008; Battisti et al., 2010; Denis et al., 2009; Potel-Alvarellos, 2009; Van Hoecke et al., 2009; Aspiroz et al., 2010; Lozano et al.,

2010; Soavi et al., 2010; Lozano et al, 2011a; Lozano et al., 2011b; van Cleef et al., 2011; Wulf et al., 2011).

2.6.8 MRSA ST398 in the general human population (Figure I-4-route 8)

In humans without animal contact, MRSA ST398 infections have been described, such as endocarditis, dermal abscesses, skin infections, sinusitis, invasive infections, wound infections and ulcers (Ekkelenkamp et al., 2006; Declerq et al., 2008; Lewis et al., 2008; Van Belkum et al., 2008; Fanoy et al., 2009; Mammina et al., 2010; Köck et al., 2013).

Possible risk factors for MRSA ST398 acquisition have been reported. First, living in a high animal density area is a risk factor for being a nasal carrier of LA-MRSA (Van Cleef et al., 2010a; Feingold et al., 2012). Second, a private farm visit increased the chance of acquisition (Bissdorff et al., 2012). Third, MRSA ST398 infected humans can act as an additional source. However, transmission of MRSA CC398 between hospitalized patients was 72% less likely than for other MRSA strains (Wassenberg et al., 2011). Fourth, Davies and colleagues (2011) remarked that not all MRSA ST398 infections should be attributed to the livestock-associated type. They emphasize that MRSA ST398 might persist in human populations without livestock contact. This was also reported in Dutch studies (Haenen et al., 2009; Lekkerkek et al., 2011). McCarthy et al. (2012) found a β-converting prophage in MRSA ST398 human isolates, which is involved in human-specific innate immune evasion, implying that some strains are human host-adapted. Finally, besides MRSA ST398, MSSA ST398 has been described in the community. This human compatible MSSA ST398 is emerging in the community independently of animal contact. The question remains, whether this strain will be able to retrieve a SCC*mec* cassette with a new human compatible MRSA ST398 strain as a result (Bhat et al., 2009; Rasigade et al., 2010; Jiménez et al., 2011; Moritz and Smith, 2011).

3 Isolation and characterization of MRSA

3.1 Isolation of MRSA

Various matrices can be sampled such as humans, animals, environment, air, carcasses and meat samples. In humans, three locations are sampled for clinical screenings: nose, throat and perineum (Bignardi and Lowes, 2009; Durai et al., 2010). In most pig studies, nasal swabs are taken. Locations such as on the skin behind the ear, the perineum, organs and faeces were also sampled. Pletinckx and colleagues (2012) reported that the best sampling location for retrieving MRSA was the skin behind the ears. However, the authors suggested that environmental contamination and not colonization of this site should be considered. For environmental sampling, dust swabs, environmental wipes or sponges were described (EFSA, 2009; Broens et al., 2010). Moreover, manure, water and feed samples were also taken (Friese et al., 2012). Three sampling methodologies exist for air sampling: impingement, filtration or open plates (Friese et al., 2012). For carcasses, the best sampling method is usage of an abrasive sponge compared to swabbing and cutting off samples (Tenhagen et al., 2011). No study reported on the best sampling location for MRSA ST398 on a carcass. Different sampling methodologies for meat samples are available. In general, “hard” meat samples (such as ribs, ears, forelimbs) are swabbed or soaked into broth and manually mixed. Ten to twenty-five grams of “soft” meat samples (such as steak, bacon and minced meat) are homogenized into broth (Lee et al., 2003; Fessler et al., 2011; Hanson et al., 2011).

It has been reported that the use of enrichment improved the MRSA isolation in clinical studies. Various studies investigated the effect of enrichment on the isolation of MRSA ST398. In general, addition of salt (6.5-7.5%) to the enrichment medium increased the MRSA ST398 detection. Staphylococci are salt-tolerant and salt inhibits the contaminating or commensal flora in the samples (Bruins et al., 2007). In Dutch studies, after the salt-enrichment step, an antibiotic enrichment step followed. Similar percentages of MRSA-positive samples were observed when comparing the Belgian study (enrichment in salt) and the Dutch studies (enrichment in salt and afterwards enrichment in antibiotics)(Graveland et al., 2009; Van Duijkeren et al., 2010; Pletinckx et al., 2009 and 2012). For environmental samples, the same salt-enrichment is used (Friese et al., 2012). Few studies have been performed on pig carcasses (see Table I-3) and in most studies, the same enrichment as described above was used. For meat samples, enrichment also occurs in salt-enriched and/or antibiotic-enriched medium (Van Loo et al., 2007; de Boer et al., 2009; O’Brien et al., 2012).

After enrichment, the samples are inoculated onto selective media. At first, agars supplemented with antibiotics were used (Wertheim et al., 2004). Throughout the years, specific chromogenic media for MRSA have been developed, such as chromID™ MRSA agar (BioMerieux), *Brilliance* MRSA agar (Oxoid) and others on which MRSA colonies have a specific color (Diederen et al., 2006; Athanasopoulos et al., 2007; Becker et al., 2002). Good performances for MRSA ST398 in human and pig nasal swabs have been reported for chromID™ MRSA, MRSA*Select*™, *Brilliance* MRSA agar and MRSA screen, but not for Oxacillin-screening agar (Graveland et al., 2009; Giotis et al., 2011; Verkade et al., 2011; Pletinckx et al., 2009 and 2012).

3.2 Confirmation of MRSA (ST398)

Once suspect colonies are obtained, biochemical tests or Polymerase Chain Reaction (PCR) protocols can be used for the confirmation of MRSA. At present, a lot of (real-time) PCRs exist for the confirmation of MRSA. However, only three MRSA ST398 specific PCRs have been described: one based on the *sau*1*hsd*1 gene (encodes a restriction modification system) and *mec*A gene, one based on four ST398 specific genes and another real-time PCR based on a 124bp ST398 specific sequence (Stegger et al., 2011; van Wamel et al., 2010; van Meurs et al., 2012).

3.3 Characterization of MRSA

3.3.1 Multilocus Sequence Typing (MLST)

During Multilocus Sequence Typing (MLST), single nucleotide variations in internal fragments of seven housekeeping genes are detected. Housekeeping genes belong to the core genes of the genome and are essential for the survival of a bacterium. Changes in these genes occur slower and equal to long periods of evolution. For *S. aureus*, the seven genes are: Carbamate kinase (*arc*C), Shikimate dehydrogenase (*aro*E), Glycerol kinase (*glp*F), Guanylate kinase (*gmk*), Phosphate acetyltransferase (*pta*), Triosephosphate isomerase (*tpi*) and Acetyl coenzyme A acetyltransferase (*yqi*L) (http://www.mlst.net; Trindade et al., 2003).

MLST is a PCR based method during which the internal fragments of the genes (500bp) are amplified and subsequently sequenced (Enright et al., 2000). Each allele receives a number and a numeric code is obtained per strain. Based on this numeric code, each strain can be classified into a sequence type (ST). All MRSA ST398 strains have 3-35-19-2-20-26-39 as

numeric code. At present, a database is available with all obtained alleles for each housekeeping gene (http://www.mlst.net), which makes comparisons much easier. Closely related STs (one allele difference = single locus variant or two alleles difference = double locus variants) can be classified in a clonal complex (CC). The CC receives the name of the most ancestral sequence type (Enright et al., 2002). For example, CC398 consists of sequence types ST398, ST541, ST572, ST753, ST1965, ST1966, ST1967, ST1968, ST1969 and others of which ST398 is considered as the ancestral type (Lim et al., 2012; Porrero et al., 2012).

MLST is a standardized, objective and highly reproducible method. Moreover, a standard nomenclature of CCs and STs is used and comparison between laboratories is possible. It is a good technique for population studies (bacterial population structure and evolution) However, MLST encounters a high cost and a low throughput, is time consuming and has only a moderate discriminatory power (Trindade et al., 2003; Urwin et al., 2003; Maiden, 2006; Strommenger et al., 2006b; Deurenberg and Strobberingh, 2008; Stefani et al., 2012).

3.3.2 *Spa* typing

Another PCR-based and sequence based method is *spa* typing (Frenay et al., 1996). This technique involves amplification and sequence analysis of the repeats (24 bp in length) in the X region of the protein A gene (Figure I-6). This method takes into account point mutations in the repeat, but also the number of repeats. After sequencing, each repeat receives a number, resulting in a numeric code which identifies the *spa* type. Besides software (Staphtype®), an international database is also available in which all reported *spa* types and their numeric codes can be found (http://spaserver.ridom.de). At present, a lot of *spa* types have been described in MRSA ST398 of which the most pre-dominant are t011, t034 and t108 (EFSA, 2009; Smith and Pearson, 2011; Vanderhaeghen et al., 2010a). It has been reported that the repeat region evolves really fast and it is expected that additional types will arise (Boye and Westh, 2011; Szabó et al., 2012).

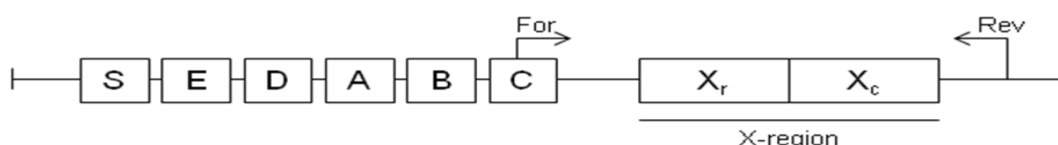

Figure I-6 Overview of the *spa* gene map. Boxes indicate segments of the gene coding for the signal sequence (S), the immunoglobulin G-binding regions (A–D), a region homologous to A–D (E), and the COOH terminus (X), which includes the repeats region (X_r) and the cell wall attachment sequence (X_c). Forward (For) and reverse (Rev) primers for *spa* typing are indicated (Shopsin et al., 1999).

Spa typing is a rapid, less expensive, less time-consuming, standardized and objective method. This technique employs a standard nomenclature. A software package and international database are available. Moreover, this method is internationally reproducible and has a higher discriminatory power compared to MLST. With this method, short-term and long-term evolution in a restricted area can be studied. Nevertheless, misclassification of a small number of lineages has been reported and use of an additional method is suggested (Shopsin et al., 1999; Oliveira et al., 2001; Strommenger et al., 2006b; Deurenberg and Stobberingh, 2008; Ikawaty et al., 2009; Rasschaert et al., 2009; Schouls et al., 2009; Furuya et al., 2010; Boye and Westh, 2011; Stefani et al., 2012).

3.3.3 Multilocus Variable-number tandem-repeat Analysis (MLVA)

A third PCR-based method is Multilocus Variable-number tandem-repeat Analysis (MLVA). MLVA detects the number of repeats in the repeat region of different selected genes. After amplification, two detection methods are available: gel electrophoresis and capillary electrophoresis. Subsequent to detection, the length of the repeat region is determined. This length is divided by the repeat length. In such way, the total number of the repeats of which a repeat region consists, is determined. Based on the number of repeats, a number is assigned to each repeat region and as a result a digit code per strain is obtained (Figure I-7).

Different gene schemes have been tested for MRSA (Sabat et al., 2003; Francois et al., 2005; Ikawaty et al., 2008; Pourcel et al., 2009; Schouls et al., 2009). For MRSA ST398 typing, Rasschaert and colleagues (2009) reported on five highly discriminatory genes (*clf*A, *clf*B, *sdr*C, *sdr*E and SIRU21). To date, MLVA typing has scarcely been used in MRSA ST398 studies.

MLVA is a cheap and rapid method. It has a high throughput and its discriminatory power ranges between that of *spa* typing and PFGE (see further). As in *spa* typing, this method can be used for short-term evolution studies. When using capillary electrophoresis, the obtained data is unambiguous and can be shared between laboratories. However, various genes have been used for MLVA and no standard protocol is available. In addition, no standard nomenclature is existing (Tenover et al., 2007; Rascchaert et al., 2009; Schouls et al., 2009; Holmes et al., 2010; te Witt et al., 2010).

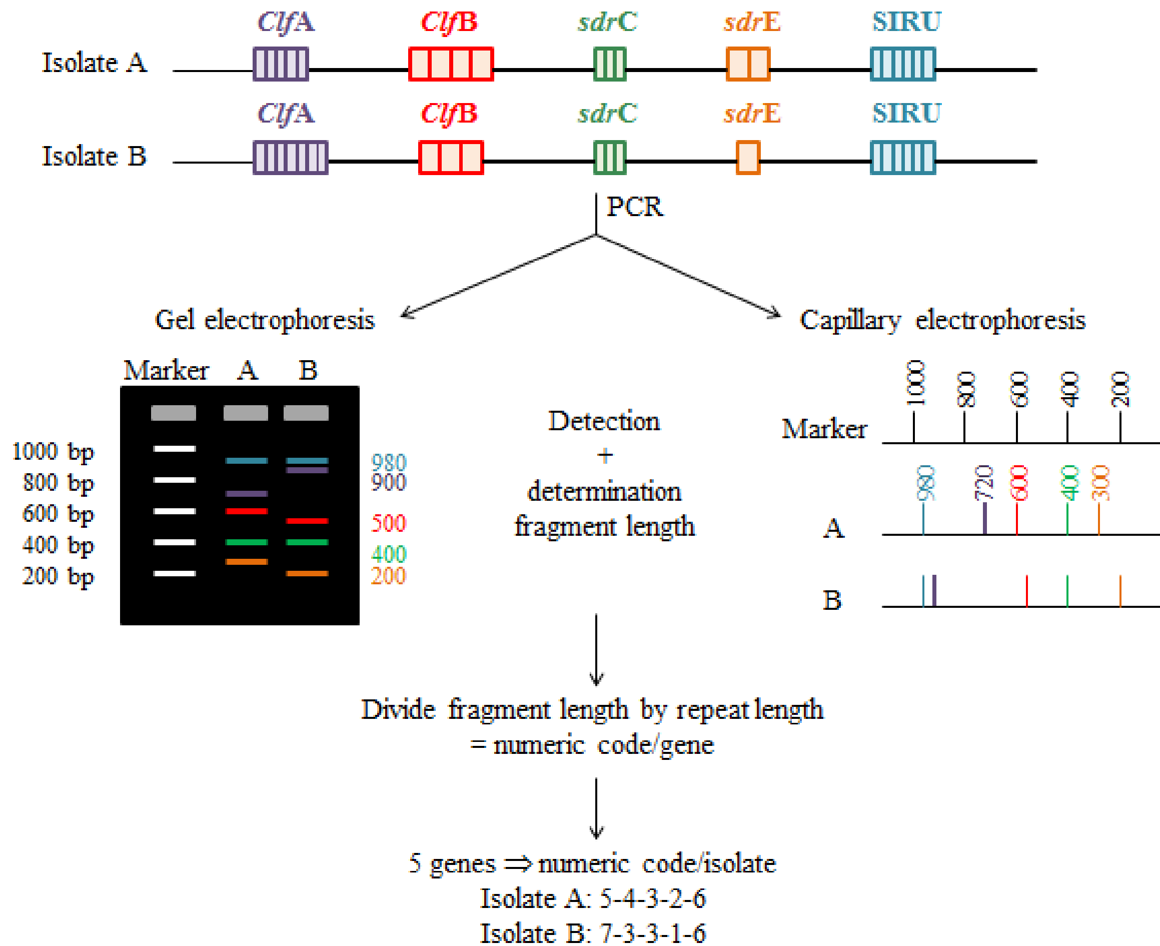

Figure I-7 Overview of MLVA typing of two isolates. After PCR, detection of the amplicons happens with gel or capillary electrophoresis. Using a marker, the length of the repeat region is determined. Dividing the obtained length by the repeat length, results in a numeric code per gene. After analyzing five genes, a numeric code per isolate is obtained.

3.3.4 SCC*mec* typing

As mentioned before, acquisition of the SCC*mec* cassette results in methicillin resistance. As mentioned in section 2.1., a SCC*mec* consists of a *mec*A gene complex, a *ccr* gene complex and three joining regions, which can be used to determine the SCC*mec* cassette type, present in an MRSA isolate. Due to the variation in SCC*mec* types, different PCR-based methods have been described throughout the years. However, seen the diversity and subtypes, none of the described methods is able to identify all cassettes and non-typeable cassettes have often been reported. In general, two approaches for SCC*mec* typing have been used. At first, the methods detected loci, specific for SCC*mec* types I, II, III and IV (Oliveira and De Lancastre, 2002; Zhang et al., 2005; Milheiriço et al., 2007). More recently, the *ccr* (1 to 5) and *mec* (A, B, C2) complexes are identified. Afterwards, the result are combined to determine the

SCC*mec* type (Table I-1). In addition, detection of loci in junkyard regions can help to determine the subtype (Kondo et al., 2007).

An international working group provided a standard nomenclature for the SCC*mec* types (www.sccmec.org). However, no standardized protocol was proposed and available for SCC*mec* typing. The use of diverse PCRs results in a relatively high cost and low throughput of samples. Moreover, this method is not as discriminatory as the others (Oliveira and de Lancastre, 2002; Francois et al., 2004; Berglund et al., 2005; Zhang et al., 2005; Kondo et al., 2007; Milheiriço et al., 2007; Deurenberg and Stobberingh, 2008; Chen et al., 2009; Rasschaert et al., 2009).

3.3.5 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) is not a PCR based method. During PFGE, bacterial cells are immobilized after embedding in agarose and after cell lysis, the whole genome becomes available. Restriction of the whole genome occurs with a "rare cutter" endonuclease, which had only few cutting sites in the genome. Different enzymes are available and each enzyme has a specific cutting site, such as CCCGGG for the enzyme *Sma*I. As a result, restriction generates large DNA fragments between 10 and 1000 Kb. Detection of these large fragments occurs in an electric field that is periodically changed, allowing efficient separation of the DNA fragments. In general, the obtained patterns are analyzed using an Unweighted Pair Group Method using Averages (UPGMA) clustering algorithm. At present, international PFGE databases and a standardized protocol (HARMONY) are available where profiles can be submitted and compared (Murchan et al., 2003). PFGE with *Sma*I restriction has long been considered as the gold standard for *S. aureus* and MRSA. But, MRSA ST398 appeared non-typeable using this method. Bens and colleagues (2006) reported that MRSA ST398 carries an unknown restriction/methylation system. As a result the *Sma*I restriction site is methylated, inhibiting *Sma*I to cut the genome. Other restriction enzymes, such as *Cfr*9I, *Bst*ZI and *Apa*I. are used instead for the typing of LA-MRSA (Rasschaert et al., 2009; Argudin et al., 2010; Bosch et al., 2010).

PFGE is able to detect more recent evolution in strains and genetic changes can lead to differences in fragments. However, the detected differences remain uncharacterized (Enright and Spratt, 1999; Peacock et al., 2002). PFGE is highly discriminatory, commonly used, reproducible and standardized. Nevertheless, as the analysis of the patterns is still subjective,

variation in the results might occur. In addition, PFGE is a technically demanding and time-consuming technique (Trindade et al., 2003; Strommenger et al., 2006b; Deurenbergh and Stobberingh, 2008; Rasschaert et al., 2009; Stefani et al., 2012).

3.3.6 Antimicrobial susceptibility typing

3.3.6.1 Phenotypic analysis

To gain more insights into the characteristics of bacterial strains, their resistance to antimicrobial agents may be determined. At present, two standardized methods are available: disk diffusion tests and Minimal Inhibitory Concentration (MIC) determination. When performing disk diffusion, a bacterial suspension is applied onto a universal growth medium (for example Mueller Hinton Agar) and disks containing a fixed concentration of an antimicrobial agent are applied. After incubation, the halo around the disk is measured and according to interpretation tables, a strain is considered sensitive, intermediate or resistant.

When determining the MIC, a bacterium is exposed to different, mostly clinical relevant, concentrations of antimicrobial agent. The lowest concentration that inhibits the bacterium is considered the MIC (CLSI, 2010). For the detection of MRSA, the antibiotics oxacillin and cefoxitin are often used (Chambers, 1997; Corrente et al., 2007; Aarestrup and Skov, 2010). In MRSA ST398 strains, acquired resistance to tetracyclin, trimethoprim, macrolides, lincosamides, aminoglycosides, chloramphenicol and fluoroquinolones was observed (Durai et al., 2010; Vanderhaeghen et al., 2010a). A large variety in antibiotypes has been reported (Kadlec et al., 2009).

After antimicrobial susceptibility testing, an adjusted treatment for bacterial infections can be used. In addition, the evolution of antimicrobial resistance in strains can be studied. Still, the method is time-consuming but at present, automated systems such as Vitek and DiversiLab® are available.

3.3.6.2 Genotypic analysis

When a bacterium becomes resistant to antimicrobial agents, this is due to mutations in the bacterial genome or the acquisition of resistance genes. To date, different molecular techniques have been described to determine these genes. However, presence of a resistance gene does not necessarily mean that the bacterium is resistant to that antibiotic (Strommenger

et al., 2003; Schnellmann et al., 2006). Table I-4 shows an overview of the resistance genes that have been described in MRSA ST398 (Kadlec et al., 2012).

Table I-4 Overview of antimicrobial resistance genes which have been reported in MRSA ST398 (Kadlec et al., 2012).

Antibiotic group	Gene	Resistance mechanism
Penicillins	*bla*Z	Enzymatic inhibitor
β-lactams	*mec*A	Altered PBP
Trimethoprim	*dfr*A, *dfr*D, *dfr*G, *dfr*K	Resistant dihydrofolate reductase
Tetracyclin	*tet*K, *tet*L, *tet*M	Active efflux Ribosome protective protein
Macrolides, lincosamides, streptograminB	*erm*(A), *erm*(B), *erm*(C), *erm*(T)	rRNA methylation
Macrolides (14-membered)	*msr*(A)	Active efflux through ABC transporter
Streptogramin A, lincosamides, pleuromutilins	*vga*(A) variants, *vga*(C), *vga*(E)	Active efflux through ABC transporter
Lincosamides	*lnu*(A)	Enzymatic inhibition through nucleotidylation
Phenicols	*fex*A	Active efflux
Phenicols, lincosamides, oxazolidinones, pleuromutilin, streptogramin A	*cfr*	rRNA methylation
Gentamicin, kanamycin, tobramycin, (amikacin)	*aac*A-*aph*D	Enzymatic inactivation through acetylation and phosphorylation
Kanamycin, neomycin	*aad*D	Enzymatic inactivation through adenylation
Kanamycin, neomycin, amikacin	*aph*A3	Enzymatic inactivation through phosphorylation
Spectinomycin	*spc*	Enzymatic inactivation through adenylation
Apramycin	*apm*A	Enzymatic inactivation through acetylation
Mupirocin	*mup*A	Mupirocin insensitive isoleucyl-tRNA synthase

3.3.7 DNA microarray analysis

DNA microarray analysis consists of different steps. First, DNA is isolated, amplified and labeled (for example by incorporation of biotin-16-dUTP). Subsequently, this labeled DNA is hybridized with a chip on which target genes or probes are fixed. When a certain gene is located on the strain DNA, hybridization occurs and a signal is observed. Possible target genes are antimicrobial resistance genes, entero- and exotoxin genes and others. To date, this

method was used to compare MRSA ST398 strains originating from pigs and humans and pigs alone (Kadlec et al., 2009; Hallin et al., 2011; Jamrozy et al., 2012).

The rapid microarray technology has the potential to detect an almost unlimited number of genes within a single reaction. Moreover, commercial and automated platforms are available. However, this technology is expensive and requires specific equipment (Cuzon et al., 2012).

3.3.8 Whole genome sequencing (WGS)

A new approach in many studies is whole genome sequencing (WGS). In the 1970s, the first method for genome sequencing has been developed. Throughout the years, better DNA preparation protocols and automated platforms have been developed which increased the use of WGS (Mardis, 2011; Dunne et al., 2012; Köser et al., 2012). To date, the genome of 49 MRSA CC398 strains has been sequenced (Schijffelen et al., 2010: Price et al., 2012; Golding et al., 2012).

WGS is the ultimate tool to discriminate between closely related strains and eases high-resolution phylogenetic reconstruction (te Witt et al., 2010; Fitzgerald, 2012; Dunne et al., 2012). Nevertheless, equipment for WGS is expensive and WGS is considered impractical for routine (Dunne et al., 2012; Köser et al., 2012; Török and Peacock, 2012).

3.4 Molecular epidemiology

Upon the first description of MRSA ST398, methods such as MLST and PFGE with *Sma*I restriction were used to characterize this new MRSA type. Later, other methods such as *spa* or SCC*mec* typing were also used for identification.

Epidemiology is the study of determinants of health, disease, and productivity in populations of humans, plants or animals (Zadoks and Schukken, 2006). Molecular epidemiology involves the use of molecular methods to study possible transmission routes of a bacterium, relationships between isolates and others. The definition of molecular epidemiology does not include identification of isolates (Foxman and Riley, 2001; Zadoks and Schukken, 2006). When studying the molecular epidemiology of isolates, a combination of two or more typing methods can be useful. It has been reported that combining methods increases the discriminatory power. For example, the discriminatory power, reported by Rasschaert and colleagues (2009) was 0.74 for *spa* typing and 0.81 for MLVA typing. Combining *spa* and MLVA typing resulted in a discriminatory power of 0.87.

To date, in many studies, molecular tools were used for MRSA ST398 identification (MLST, *spa* typing, PFGE). However, few studies on the molecular epidemiology of MRSA ST398 in pigs have been performed. The early studies on MRSA ST398 involved molecular typing of limited numbers of isolates and in most cases the use of MLST, *spa* typing and PFGE with *Sma*I restriction (Huijsdens et al., 2006; Van Duijkeren et al., 2008; Denis et al., 2009; Weese et al., 2010a). When it was reported that other restriction enzymes were able to generate a fingerprint in PFGE, some research groups used these other enzymes (Bosch et al., 2010; Huber et al., 2010; Lozano et al., 2011a; Espinosa-Gongora et al., 2012b). Besides MLST, *spa* typing and PFGE, methods such as SCC*mec* typing, determination of antibiotic resistance or virulence genes, micro-array or whole genome sequencing have been used for molecular epidemiology (Schijffelen et al., 2010: Hallin et al., 2011; Jamrozy et al., 2012; Price et al., 2012). MLVA typing has scarcely been used in the molecular epidemiology of MRSA ST398.

4 Belgian pig production chain

4.1 Pig farms

In the EU, approximately 1875 million livestock animals are present of which 155 million pigs. Belgium is situated in the top ten of the European member states for pig stock. The Belgian pig stock contains approximately 6 million pigs (1.5 million piglets up to 20 kg; 1.2 million pigs between 20-50 kg; 2.6 million finishers >50kg and 0.5 million sows). From 1999 to 2011, the number of pigs decreased from approximately 7.5 million to 6 million pigs (Figure I-8) (Platteau et al., 2010a and b; Platteau et al., 2012).

The pig sector is mainly situated (94%) in Flanders. In 2011, approximately 5000 pig farms were present in Flanders. Around 3000 farms are specialized pig farms (exclusively pigs). The remaining farms are considered mixed farms, i.e. farms where more than one livestock species is present (Platteau et al., 2012). At present, there are approximately 200 pig-poultry and 3000 pig-cattle farms (personal communication with Animal Health Care Flanders (DGZ), 2012).

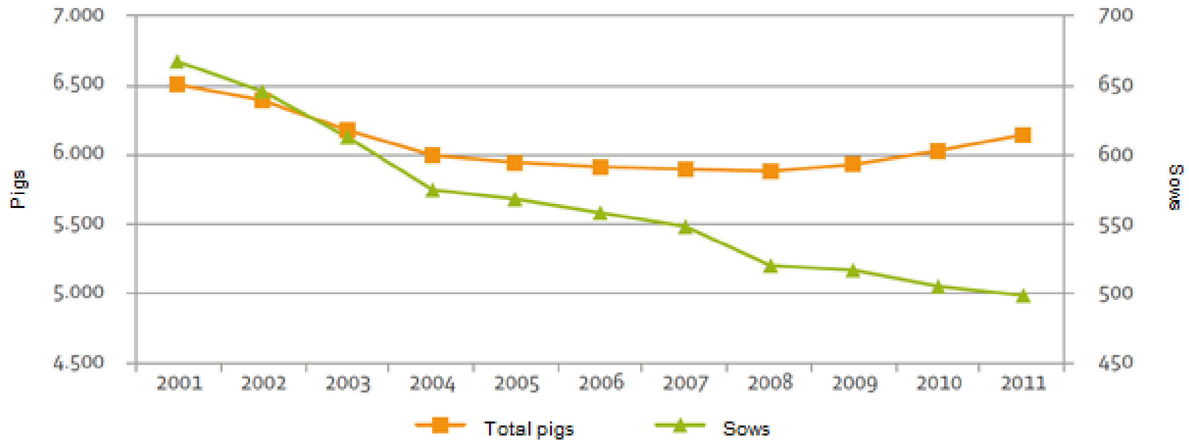

Figure I-8 Evolution of the pig stock in Belgium from 1999 to 2011, per 1000 units (Platteau et al., 2012).

There are three farm types: 1) closed or farrow-to-finish farms; 2) farrowing farms and 3) open, rearing or finishing farms. On a farrow-to-finish farm, sows are present and piglets are born on site. The piglets stay at the same farm until slaughter age. On a farrow farm, the piglets are moved to another farm after weaning. On a finishing farm, the animals arrive at the farm after weaning where they reside until slaughter age. A farm consists of certain units, depending on the farm type (Figure I-9).

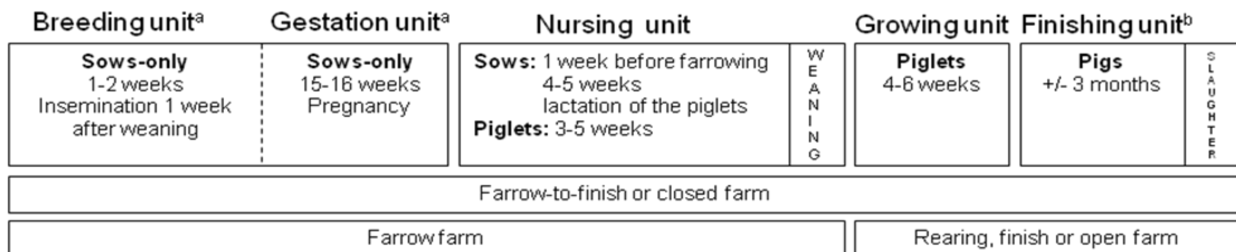

Figure I-9 Overview of the units that can be present on a farrow-to-finish, a farrow and a finish farm. [a] Both units can be separate or together, b On some farms, two finishing units are present (after a stay of 4 weeks in finishing unit 1, the animals are transferred to a second unit).

Up to five units can be present on the farm. When present on the farm, sows reside in three units (breeding, gestation and nursing unit). Piglets can be found in the nursing, growing and finishing unit (Figure I-9). In the breeding unit, sows undergo insemination. After approximately one week, the sows are moved to the gestation unit, where they reside for 15 to 16 weeks (= gestation). On most farms, both breeding and gestation unit are one large unit. Sows reside in separate pens and sometimes together in groups. However, from January 2013 onwards, it will be obligatory to keep sows in groups. One week before farrowing, the sows are moved to the nursing unit, where they reside in separate pens. After farrowing, the sows suckle their offspring until weaning. The length of the lactation period depends on the

production cycle (see further). At weaning, the sows and their offspring are separated. The sows enter the breeding unit, where they undergo insemination after approximately one week. The piglets go to the growing unit. After 4 to 6 weeks (depending on the production cycle and weight gain), the pigs are moved to the finishing unit, where they stay until slaughter age.
The production cycle of a farm depends on the sows' cycle as described above. A sows' cycle lasts 21 weeks (approximately 147 days: 7 days between weaning and insemination, 114 days of gestation and 26 days of lactation). The goal of sow management is to inseminate a large group of sows at the same time, to wean a large group of piglets at the same time and to obtain a large group of pigs for slaughter at the same time. This results in a more standardized scheme for the farmer. For example, during a three-weeks production cycle, every three weeks, a group of sows farrows. In this production cycle, seven sow groups are present in total to maintain a constant occupation of the units. In analogy, a one-, two-, four-, five-, six- and seven-weeks cycle exists. Depending on the production cycle, animals reside shorter or longer in the units.

4.2 Slaughterhouse

At slaughter age, the pigs are transported to the slaughterhouse, where they reside between 1 to 12 hours in the lairage. From the lairage on, the animals are guided towards the slaughter line, which is called the dirty area of the slaughterhouse. After stunning, stabbing of the pig occurs. Successively, the carcass bleeds out, is scalded and dehaired. Subsequently, the carcass undergoes singeing after which brushing of the carcass occurs sometimes. After this, the carcass enters the clean area of the slaughterhouse. In this clean area evisceration of the carcass occurs during which the internal organs are removed. After evisceration, cooling of the carcasses (two-three hours) happens. After cooling, the carcasses are deheaded and both carcass halves go to the meat processing area or stay intact (Figure I-10) (Houf, 2004; Claeys et al., 1998).

In Europe, approximately 22 million tons of pork were produced in 2008. In 2011, approximately 11.8 million Belgian pigs were slaughtered. This equals to approximately 1.1 million ton of pork, which is around 5% of the total European pork meat. The average European pork consumption is 20 million tons per year. In Flanders, approximately 10 kg pork per person was consumed on average per year. The self-sufficiency of Belgian pork was 242% in 2007 (most recent number), which means that the pork production is higher than

pork consumption i.e. Belgium is an important pork exporter (Platteau et al., 2010a and b: Platteau et al., 2012).

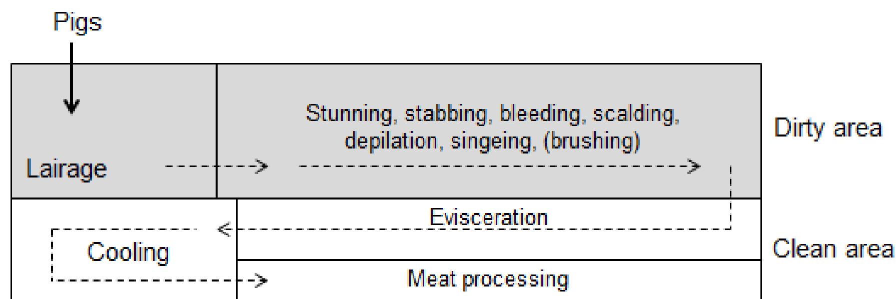

Figure I-10 Scheme of a slaughterhouse (adapted from Van Cleef et al., 2010b).

4.3 Consumption of antimicrobial agents in Belgian livestock/pig farming

Antimicrobial agents are administered to livestock animals via injection (individual treatment) or through feed or water premixes (group treatment). According to the Belgian Veterinary Surveillance of Antimicrobial Consumption (BELVET-SAC) report of 2011, 241 tons of pharmaceuticals and 57 tons of premixes were consumed for veterinary use (Dewulf et al., 2012). Approximately 99% of antimicrobial premixes are used in pig feed (Dewulf et al., 2012).

Over a five-year period, following antimicrobial classes have been primarily used in Belgian livestock farming: sulphonamides and trimethoprim (31%), tetracyclines (26%), penicillins (25%) and macrolides (7%). More antimicrobial classes have been used in antimicrobial pharmaceuticals than in feed premixes. However, in feed premixes more sulphonamides and trimethoprim and less macrolides were used in comparison with the antimicrobial pharmaceuticals (Dewulf et al., 2012). For Belgium, no data are available on the antimicrobial use of the whole pig farming sector. However, Callens and coworkers (2012) collected data on 50 Belgian pig farms. Following antibiotics were used for oral application at group level: colistin (30.7%), amoxicillin (30.0%), trimethoprim-sulfonamides (13.1%), doxycycline (9.9%) and tylosin (8.1%). The injectable antimicrobials were tulathromycin (45.0%), long-acting ceftiofur (40.1%) and long-acting amoxicillin (8.4%).

Administration of antimicrobial agents can occur at various stages in the pig lifespan and in most cases group treatments occur. In the nursing unit, animal treatment can occur immediately after birth and upon castration (sometimes treatment of both males and female

pigs), whereas in the growing and finishing unit treatment occurs upon arrival and around slaughter age, respectively.

4.4 MRSA Surveillances

From 2005 onwards, many national studies have been conducted on MRSA ST398 presence in livestock animals. To determine the MRSA prevalence in food animals and the MRSA clone(s) in these animals, the Task force on Zoonoses data collection of the European Food Safety Authority (EFSA) suggested additional sample collection for MRSA in the *Salmonella* baseline survey in pigs. During the MRSA baseline study, five dust samples were collected on pig breeding and fattening holding. All obtained isolates were *spa* typed and on a selection, MLST occurred. In Belgium, a prevalence of 40.0% and 35.9% were found in breeding and production holdings, respectively. *Spa* types t011, t034, t567, t1451 and t2370 were isolated (EFSA, 2007; EFSA, 2009).

Besides EFSA, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) conducts MRSA surveillances. In 2007, a survey on MRSA prevalence in pig farms and pigs was conducted where faecal samples were taken (30 samples per farm, 50 farms). A farm prevalence of 68% was observed (FASFC, 2008). Crombé et al. (2012a) reported on a nasal prevalence of 44% on pig level and a prevalence of 68% on farm level (94% of open and 47% of closed farms). Remarkably, in open farms, more MRSA positive animals were observed. The isolates belonged to ST398 and *spa* types t011 and t034 were detected (FASFC, 2008; Crombé et al., 2012a). The MRSA surveillance in pigs occurs every three years. To our knowledge, no results of the pig surveillance of 2010 are available. In 2011, the MRSA surveillance in poultry was conducted: 2.1% of the poultry farms were MRSA positive (0.6% were layer farms and 1.5% were the broiler farms) (FASFC, 2012).

4.5 MRSA legislation

If known, the presence of MRSA in living animals should be reported 24 hours before slaughter to the slaughterhouse by the farmer or the herd veterinarian (www.favv.be). The FASFC has described guidelines for the bacterial contamination of food and food products. The accepted amount for coagulase-positive *Staphylococcus* is ≤100-1000 cfu per gram minced meat, sausage, bacon and raw meat. When more than 10^5 cfu/g are present, the toxin production of the strains should be analyzed in 25g. No specific guidelines have been described for MRSA (ST398) (FAVV, 2010).

Chapter II. General aims

Animal-associated methicillin-resistant *Staphylococcus aureus* (MRSA) or MRSA ST398 has been described in pigs in many countries worldwide from 2005 on. Pigs are considered as a potential MRSA source for the human population.

The general aim of this PhD work was to get a better insight in the epidemiology of MRSA ST398 throughout the Belgian pig production sector by means of sampling events and typing methods.

The specific aims were as follows:

- to screen different farm types (pig, pig-poultry, pig-cattle) on the MRSA ST398 presence on those farm types and to determine whether the presence of an additional animal species has an influence on the pig prevalence and the genetic diversity of LA-MRSA within the farm types (Chapter 3).
- to determine potential MRSA sources for piglets, the MRSA colonization age of piglets and the evolution of the piglet MRSA carriership after a longitudinal molecular study on four selected farrow-to-finish farms (Chapter 4a and 4b).
- to determine the best sampling location for MRSA on a pig carcass (Chapter 5a).
- to determine the MRSA presence on pork (Chapter 5b).

Chapter III. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 in pig farms and multispecies farms

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1 Abstract

During the last few years, methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 has been isolated frequently from livestock, especially from pigs and to a lesser extent from cattle and poultry. To gain insight into the distribution of this bacterium in pig farms versus multispecies farms, 30 Belgian farms (10 pig, 10 pig/poultry and 10 pig/cattle farms) were screened for the presence of MRSA. On each farm, 10 nasal swabs were taken from pigs. When present, cattle (n=10) were sampled in the nares and poultry (n=10) in the nares, earlobes and cloaca. A selection of the obtained isolates were further characterized using multilocus sequence typing (MLST), *spa* typing, SCC*mec* typing, pulsed field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA) and antimicrobial susceptibility testing. On 26 out of 30 farms, MRSA was isolated from pigs. Furthermore, MRSA was also isolated from poultry and cattle on one pig/poultry and five pig/cattle farms, respectively. All tested MRSA isolates belonged to ST398. Eight *spa* types (t011, t034, t567, t571, t1451, t2974, t3423 and t5943) were detected, among which t011 predominated. SCC*mec* cassettes type IVa and V were present in 20% and 72% of the isolates, respectively. When combining the results of the two remaining typing methods, PFGE and MLVA, eighteen genotypes were obtained of which one genotype predominated (56% of the positive farms). All MRSA isolates were resistant to tetracyclin. Resistance to trimethoprim, aminoglycosides, macrolides, lincosamides, fluoroquinolones and chloramphenicol was also observed.

In conclusion, MRSA was isolated more often from pigs in comparison to poultry and cattle. No significant difference between the farm types was observed. Additionally, a wide variety of MRSA ST398 strains was found within certain farms when combining different typing methods.

2 Introduction

In 2005, decades after the discovery of the hospital-associated and community-associated MRSA, a new MRSA type was isolated from pigs and pig farmers in the Netherlands and was named livestock-associated MRSA or LA-MRSA (Voss et al., 2005). Molecular typing with multilocus sequence typing (MLST) classified this clone into clonal complex (CC) 398 with sequence type 398 (ST398) as basic type (Vanderhaeghen et al., 2010a). Additionally, MRSA ST398 has been related to different *spa* types, in particular t011, t034 and t108 (EFSA, 2008). Resistance to the antibiotics used in livestock farming such as tetracyclin, trimethoprim, and aminoglycosides was found in LA-MRSA isolates (EFSA, 2008; Vanderhaeghen et al., 2010a).

LA-MRSA has been reported worldwide, including in Belgium, and is known to colonize humans and livestock animals such as pigs, cattle and chickens (Vanderhaeghen et al., 2010a; Weese and van Duijkeren, 2010). The presence of MRSA ST398 in animals and humans is of concern for human and food safety, as colonized animals may act as a reservoir. This might pose a possible threat for human infection. For exposed persons, the transmission risk is high, resulting in a certain risk for developing MRSA infections (Denis et al., 2009; Köck et al., 2010). Within a farm, transmission of MRSA ST398 is expected to occur between animals, the environment and humans. In addition to pig-only farms, Belgium has multispecies farms (pig/cattle and pig/poultry farms). The presence of an additional animal species on a farm might play a role in the transmission of MRSA and have an influence on the MRSA status of the animals present on the farm. In this study, 30 Belgian farms were screened for the presence of LA-MRSA. The aims of the study were i) to gain insight into the LA-MRSA presence on different farm types, ii) to examine the genetic diversity of LA-MRSA within those farm types, iii) to determine whether other CCs than CC398 are present in cattle and poultry on multispecies farms and iv) to determine any correlation between pigs and other species reared on one farm.

3 Materials and Methods

3.1 Sample collection and farm management

Three different farm types were included in this study being pig farms, pig/poultry farms and pig/cattle farms. From March to June 2009, 30 Belgian farms (ten farms belonging to each

farm type) were screened once. All farms were located in Flanders (the northern part of Belgium). The farms were randomly selected by Animal Health Care of Flanders (DGZ) and Catholic University College of South-West-Flanders (KATHO). Inclusion factors for the farms were: located in Flanders, preferably farrow-to-finish farms and multispecies farms required the presence of cattle or poultry besides pigs (Table III-1). Two pig farms had a specific pathogen free (SPF) status. This status indicates that the farmers use strict biosafety protocols and eradication programmes for certain pathogens (but not MRSA), resulting in healthier pigs and better production numbers. On multispecies farms, there was no direct contact between the different animal species. The contact time of the farmers with the animal species differed per species (Table III-1). Contact time with pigs was approximately three to four hours (nursing unit + walk through barn), whereas contact time with dairy cattle was around two to four hours (milking + walking through barn). On pig/poultry farms, farmers spent less than one hour in the poultry barns (walking through). Before entering the poultry barn, the farmer always changed boots and boot dipping occurred in antiseptic products, such as Virocid, ammonium and others. Prior to entering the milking unit, footwear and clothes were often changed.

Cotton swabs were brought into 3 ml salt-enriched (6.5%; Sodium chloride; 1.06404; Merk, Darmstadt, DE) nutrient broth (NB; CM0001; Oxoid, Basingstoke, UK). On each of the 30 farms, nasal swabs were obtained from 10 pigs (12-13 weeks old). On pig/poultry farms, nasal swabs were derived from 10 chickens (at least 5 weeks old); additionally, samples from the earlobe and cloacae were taken from these 10 chickens using the same swab. On pig/cattle farms, nasal swabs were taken from 10 cows (6 to 12 months old). The 10 pigs were located in one barn. Depending on the amount of pens, one to two pigs per pen were randomly sampled using a table of random numbers. The cattle and chickens were also randomly chosen from one barn using the same table. The swabs were stored into the salt-enriched NB, transported to the laboratory and processed immediately upon arrival.

3.2 Sample processing

After overnight incubation of the swabs in salt-enriched NB (18-20 h, 37°C), 1µl was plated onto a chromogenic selective medium for MRSA (Chrom-ID^{TM} MRSA; BioMérieux, Marcy l'Etoile, FR). One suspect colony, obtained after incubation (18-20 h, 37°C), was purified by plating onto the MRSA-selective medium.

Table III-1 Characteristics of the 10 pig, 10 pig/poultry and 10 pig/cattle farms (n=30)

	Type of farm	Number of animals per farm	Distance pigs -other animals	Location	Farmer contact		
					Times/day	Time (hours)	Procedure upon barn entry
Pigs	FF (n=29) FI (=1)	2000-3000 1000-2000	-	Closed barn	1	3-4	Farm clothes and shoes
Poultry	Layers (n=4)	Layers: 7000 to 30000	50-100m	Closed barn	1	<1	Change of boots + boot dipping
	Broilers (n=6)	Broilers: 25000 to 75000					
Cattle	Dairy (n=10)	10-60	50-100m	Closed barn (n=4) Outside (n=4) Both (n=2)	2	2-4	Change of shoes/clothes

FF, farrow-to-finish farm; FI, finishing farm

After plating on tryptone soy agar (TSA; CM0131; Oxoid, Basingstoke, UK) and overnight incubation at 37°C, pure isolates were stored at -20°C in brain-heart infusion broth (BHI; CM0225; Oxoid, Basingstoke, UK) supplemented with glycerol (15% wt/vol; Fisher Scientific, Leicestershire, UK).

3.3 MRSA confirmation

DNA was extracted from each isolate according to the method of Strandén et al. (2003) and then stored at -20°C until further use. For confirmation, a multiplex PCR that detects the presence of *mec*A, *nuc* and a *S. aureus* specific signature sequence of 16S rDNA was used as described by Maes et al. (2002). During the present study, a farm was considered positive when MRSA was isolated from at least one animal. No retrieval of MRSA from the animals of a farm does not imply that a farm is MRSA-negative.

3.4 Statistical analysis

Statistical analysis occurred in SPSS statistics 19 (IBM, Chicago, IL, US). A Spearman-rank test was performed to test the hypothesis that the number of positive cattle and poultry is correlated with the number of positive pigs on ten pig-cattle and ten pig-poultry farms, respectively. To test the hypothesis that the farm type has an effect on the pig MRSA status, a logistic regression was used on the numbers of positive/MRSA not detected animals obtained per farm type. For all analyses, a $P < 0.05$ was considered significant. To compare the diversity of genotypes, obtained per farm type, the Simpson's index of diversity (D) was calculated (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php).

3.5 Molecular typing

On all obtained MRSA isolates (n=170), *spa* typing was performed according to Ridom StaphType standard procedure (http://www.ridom.de/staphtype) and the *spa* type was determined using the Ridom StaphType software (Ridom GmbH, Würzburg, DE). Also, SCC*mec* typing was performed, based on the protocols of Milheiriço et al. (2007), Oliveira and de Lencastre (2002) and Zhang et al. (2005). Different PCR mixtures were used to discriminate SCC*mec* types I, Ia, II, III, IIIa, IIIb, IV, IVa-IVh and type V. Data were combined to obtain the SCC*mec* type. When a non-typeable SCC*mec* type was obtained, the method of Kondo et al. (2007) was used and the *mec* and *ccr* complex number was given. MLST on seven housekeeping genes was performed on isolates, representing the different *spa*

types as described by Enright et al. (2000). Allele numbers and sequence types were assigned using the *S. aureus* MLST website (http://saureus.mlst.net).

Sixty-two isolates were selected arbitrarily to include each farm and each animal species present on a farm. PFGE was performed on this selection using *Bst*ZI (Promega, Madison, WI, US) as restriction enzyme as described by Rasschaert et al. (2009). The obtained restriction profiles were analysed using the unweighted pair group method using averages (UPGMA) with the Dice coefficient (tolerance 1%, tolerance change 1% and optimization 1%). Pulsotypes were determined based on a delineation level of 97% (Bionumerics version 6.5; Applied Maths, St.-Martens-Latem, BE).

Multiple-locus variable-number tandem-repeat analysis (MLVA) was applied on the same selection of isolates (Rasschaert et al., 2009). Fragment analysis of the PCR products occurred on a 3130xl Genetic Analyzer (Applied Biosystems/Hitachi, Hitachinaka-shi, JP) together with the Genescan™ 1200LIZ® size standard (4379950, Applied Biosystems, Warrington, UK). The obtained patterns were transformed into numeric codes using the MLVA plugin in Bionumerics version 6.5. Categorical analysis was performed (tolerance: 5%) to obtain different MLVA types with a delineation level of 97%. To determine the genotype, a consensus clustering was made in Bionumerics version 6.5 using UPGMA. PFGE and MLVA results will be referred to as P and M, respectively (e.g. P-6, M-6). Combined genotypes were delineated at a 97% level and were presented in capital letters.

3.6 Antimicrobial susceptibility testing

A disk diffusion method was performed to determine the antimicrobial resistance of the selected isolates, according to the CLSI procedure (CLSI, 2010). Neo-sensitabs™ were used (Rosco Diagnostica, Taastrup, DK) with the interpretation tables of the manufacturer (based on the CLSI method). Sixteen different antimicrobial agents were tested: chloramphenicol (CLR; 60 µg), ciprofloxacin (CIP; 10 µg), erythromycin (ERY; 78 µg), fucidin (FUC; 100 µg), gentamicin (GEN; 40 µg), kanamycin (KAN; 100 µg), lincomycin (LIN; 19 µg), linezolid (LINE; 30 µg), mupirocin (MUP; 10 µg), quinupristin/dalfopristin (SYN; 15 µg), rifampicin (RIF; 30 µg), sulphonamides (SUL; 240 µg), tetracyclin (TET; 80 µg), tobramycin (TOB; 40 µg), trimethoprim (TRI; 5.2 µg) and tylosin (TYL; 150 µg). *S. aureus* ATCC25923 and a MRSA ST398 strain (MB4360), isolated during a previous study, were used as reference strains.

4 Results

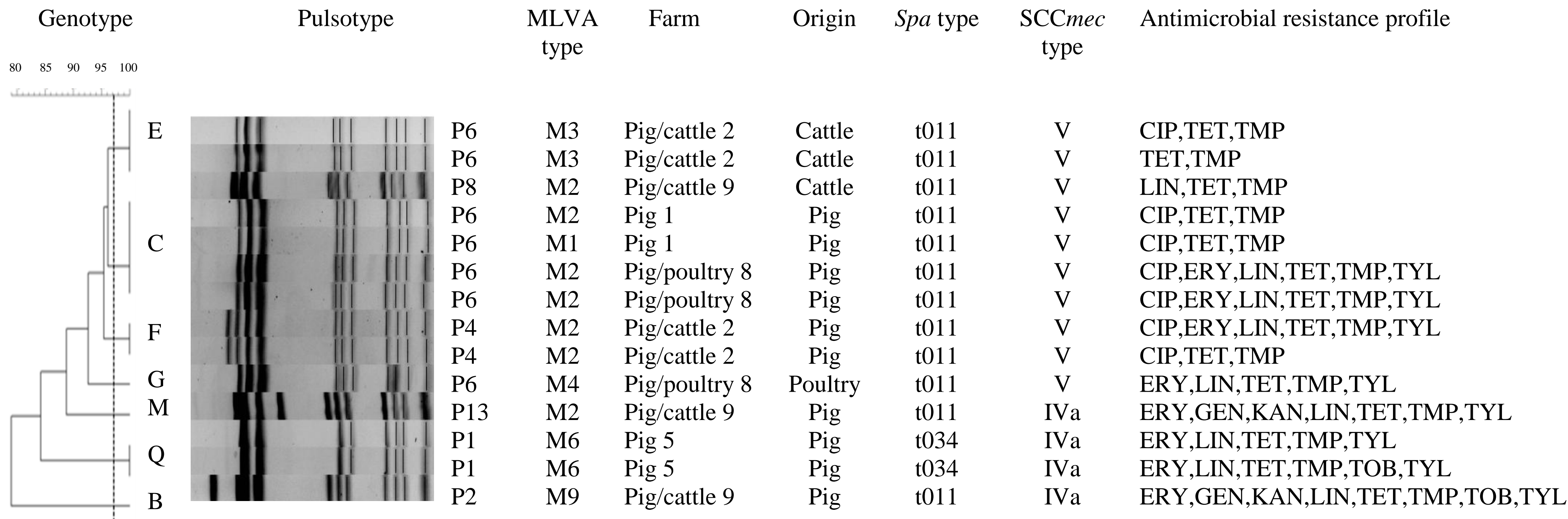
MRSA was detected in pigs on 26 out of the 30 screened farms, being ten pig farms, eight pig/poultry farms and eight pig/cattle farms. MRSA was detected once in poultry (broilers) on only one of these eight pig/poultry farms. Cattle were colonized with MRSA on five pig/cattle farms, of which four had colonized pigs. MRSA was detected more often in pigs (77%) than in poultry (2%) and in cattle (26%). We observed no correlation between the number of positive poultry or cattle and the number of positive pigs (P = 0.46 and P=0.27, respectively). In this screening study, no significant effect between the farm types was observed (logistic regression, P = 0.13).

In total, 170 isolates were obtained, of which 155 pig isolates (155/300 nasal swabs), 14 cattle isolates (14/100 nasal swabs) and one poultry isolate (1/100 nasal/earlobe/cloaca swabs). MLST typing showed that all tested isolates belonged to ST398. *Spa* typing on all isolates revealed the presence of eight different *spa* types with t011 as the dominant type (93% of the positive farms; 79% of all tested MRSA isolates) followed by s*pa* types t034 (11%; 8%) and t1451 (7%; 4%). The remaining *spa* types t567, t571, t5943, t2974 and t3423 were found on only one farm each. On 21 farms, only one *spa* type per farm was found (Table III-3). SCC*mec* typing revealed a predominance of SCC*mec* type V, occurring on 89% of the positive farms (72% of the isolates), whereas SCC*mec* type IVa occurred on 37% of these farms (20%). The SCC*mec* type of two farms remained non-typeable. Only one SCC*mec* type was found on 20 farms (Table III-2). When combining the *spa* typing results with the SCC*mec* typing, 11 combinations were found, of which the combination t011-V was predominant (81% of the positive farms and 66% of the isolates). The combinations t011-IVa, t034-IVa and t1451-V were found on 22% (13% of the isolates), 7% (8%) and 7% (4%) of these farms, respectively. The remaining combinations occurred on only one farm. On 17 farms, only one combination of a *spa*-SCC*mec* type was found.

*Bst*ZI digestion of the selected isolates revealed the presence of 14 pulsotypes with P-6 as the dominant type, which was found on 16 out of the 27 positive farms (59%; 53% of the tested isolates; data not shown). MLVA clustered the isolates into 10 MLVA types, from which M-2 appeared predominant, which was present on 22 of these farms (81%; 61% of the tested isolates; data not shown). Combining both fingerprinting techniques resulted in 18 genotypes (Table III-2). The dominant genotype C was present on 56% of the positive farms (i.e. four pig farms, six pig/poultry farms and five pig/cattle farms) and 44% of the tested isolates.

Genotype F was observed on one pig, two pig/poultry and two pig/cattle farms (19%; 10%); genotype L was found on three pig and one pig/cattle farm (15%; 8%) and genotype K was detected on one farm of every farm type (11%; 5%). The remaining genotypes occurred on one to two farms. Eleven genotypes were found on the pig farms, compared with six and nine on the pig/poultry and pig/cattle farms, respectively. The Simpson's index of diversity, which gives an indication of the genetic diversity in relation to the number of isolates per genotype, was 0.89 for pig farms, whereas 0.68 and 0.76 for pig-poultry farms and pig-cattle farms, respectively. Only one genotype was found on five pig farms, four pig/poultry farms and three pig/cattle farms. On the remaining farms, two genotypes were present, except for one pig farm and one pig/cattle farm, where three genotypes were observed (Table III-2; Figure III-1).

Antimicrobial susceptibility testing of the selected MRSA isolates revealed the presence of 16 combinations of antibiotic resistance (Table III-2). On all farms, resistance to tetracyclin was observed. Resistance to trimethoprim was seen on all but three farms (one pig and two pig/poultry farms; 92% of the tested isolates). Resistance to lincomycin was found on 74% of the positive farms and resistance to erythromycin and tylosin was found on 56% of these farms (61% and 40% of the tested isolates, respectively). On 26% of the positive farms (22% of the isolates), resistance to ciprofloxacin was observed, whereas on 33% of these farms (15%), combined resistance to gentamicin, kanamycin and tobramycin was found.



Pulsotype	MLVA type	Farm	Origin	*Spa* type	SCC*mec* type	Antimicrobial resistance profile
P6	M3	Pig/cattle 2	Cattle	t011	V	CIP,TET,TMP
P6	M3	Pig/cattle 2	Cattle	t011	V	TET,TMP
P8	M2	Pig/cattle 9	Cattle	t011	V	LIN,TET,TMP
P6	M2	Pig 1	Pig	t011	V	CIP,TET,TMP
P6	M1	Pig 1	Pig	t011	V	CIP,TET,TMP
P6	M2	Pig/poultry 8	Pig	t011	V	CIP,ERY,LIN,TET,TMP,TYL
P6	M2	Pig/poultry 8	Pig	t011	V	CIP,ERY,LIN,TET,TMP,TYL
P4	M2	Pig/cattle 2	Pig	t011	V	CIP,ERY,LIN,TET,TMP,TYL
P4	M2	Pig/cattle 2	Pig	t011	V	CIP,TET,TMP
P6	M4	Pig/poultry 8	Poultry	t011	V	ERY,LIN,TET,TMP,TYL
P13	M2	Pig/cattle 9	Pig	t011	IVa	ERY,GEN,KAN,LIN,TET,TMP,TYL
P1	M6	Pig 5	Pig	t034	IVa	ERY,LIN,TET,TMP,TYL
P1	M6	Pig 5	Pig	t034	IVa	ERY,LIN,TET,TMP,TOB,TYL
P2	M9	Pig/cattle 9	Pig	t011	IVa	ERY,GEN,KAN,LIN,TET,TMP,TOB,TYL

Figure III-1 Example of a consensus cluster of the composite data set and the PFGE fingerprints. A delineation level of 97% (dotted line) was applied to discriminate the different genotypes. (P: pulsotype, M: MLVA type, CIP: Ciprofloxacin, ERY: Erythromycin, GEN: Gentamicin, KAN: Kanamycin, LIN: Lincomycin, TET: Tetracyclin, TMP: Trimethoprim, TOB: Tobramycin, TYL: Tylosin).

Table III-2 Typing results of the MRSA isolates obtained from the different farms (CHL, Chloramphenicol; CIP, Ciprofloxacin; ERY, Erythromycin; GEN, Gentamicin; KAN, Kanamycin; LIN, Lincomycin; TET, Tetracyclin; TMP, Trimethoprim; TOB, Tobramycin; TYL, Tylosin; NT, Non-typeable). On two pig/poultry farms and one pig/cattle farm, no MRSA was isolated from the animals.

Farm type and number	Number of positive samples per farm[a]	*spa*-SCC*mec* types/farm (number of isolates)[b]	Isolate origin (number of isolates)[c]	*spa* type[c]	SCC*mec* type[c]	Genotype[c]	Antimicrobial resistance profile[c]
Pig 1	10/10	t011-V (10)	pig (2)	t011	V	C	CIP, TET, TMP
Pig 2	9/10	t567-NT (7)	pig (1)	t567	*mec*NT/*ccr*C[d]	A	TET, TMP
		t5943-NT (2)	pig (1)	t5943	*mec*NT/*ccr*C[d]	R	ERY, LIN, TET, TMP, TYL
Pig 3	7/10	t011-V (7)	pig (1)	t011	V	K	ERY, LIN, TET, TMP, TYL
			pig (1)	t011	V	H	ERY, LIN, TET, TMP, TYL
Pig 4	10/10	t011-V (7)	pig (1)	t011	V	C	ERY, LIN, TET, TMP, TYL
		t034-IVa (3)	pig (1)	t011	V	F	ERY, GEN, KAN, LIN, TET, TOB
Pig 5	10/10	t034-IVa (7)	pig (1)	t034	IVa	Q	ERY, LIN, TET, TMP, TYL
		t011-V (3)	pig (1)	t034	IVa	Q	ERY, LIN, TET, TMP, TOB, TYL
Pig 6	10/10	t011-V (10)	pig (2)	t011	V	C	ERY, LIN, TET, TYL
Pig 7	9/10	t011-IVa (9)	pig (2)	t011	IVa	D	ERY, GEN, KAN, LIN, TET, TMP, TOB, TYL
Pig 8	10/10	t011-V (9)	pig (1)	t011	V	L	GEN, KAN, TET, TMP, TOB
		t3423-IVa (1)	pig (1)	t3423	IVa	L	GEN, KAN, TET, TMP, TOB
Pig 9	5/10	t011-IVa (4)	pig (1)	t011	IVa	O	GEN, KAN, TET, TMP
		t011-V (1)	pig (1)	t011	IVa	L	GEN, KAN, TET, TMP
Pig 10	3/10	t011-V (2)	pig (1)	t011	V	J	ERY, GEN, KAN, LIN, TET, TMP, TYL
		t2974-IVa (1)	pig (1)	t2974	IVa	L	ERY, GEN, KAN, LIN, TET, TMP, TOB, TYL
			pig (1)	t011	V	C	LIN, TET, TMP
Pig/poultry 1	5/10	t1451-V (3)	pig (2)	t1451	V	C	LIN, TET, TMP
		t011-V (2)					
Pig/poultry 2	3/10	t011-V (3)	pig (2)	t011	V	C	CIP, TET, TMP
Pig/poultry 3	5/10	t011-V (5)	pig (1)	t011	V	K	CIP, TET, TMP
			pig (1)	t011	V	C	CIP, TET, TMP
Pig/poultry 4	5/10	t011-V (5)	pig (1)	t011	V	C	CIP, TET, TMP
			pig (1)	t011	V	J	CIP, TET, TMP
Pig/poultry 5	5/10	t571-NT (4)	pig (2)	t571	*mec*NT/*ccr*C[d]	P	LIN, TET
		t571-V (1)					
Pig/poultry 6	5/10	t011-V (5)	pig (1)	t011	V	C	TET, TMP
			pig (1)	t011	V	F	TET, TMP

Farm type and number	Number of positive samples per farm[a]	*spa*-SCC*mec* types/farm (number of isolates)[b]	Isolate origin (number of isolates)[c]	*spa* type[c]	SCC*mec* type[c]	Genotype[c]	Antimicrobial resistance profile[c]
Pig/poultry 7	1/10	t011-V (1)	pig (1)	t011	V	F	ERY, LIN, TET, TYL
Pig/poultry 8	5/10 (pig)	t011-V (6)	pig (2)	t011	V	C	CIP, ERY, LIN, TET, TMP, TYL
	1/10 (poultry)		chicken (1)	t011	V	G	ERY, LIN, TET, TMP, TYL
Pig/cattle 1	5/10	t1451-V (3)	pig (2)	t1451	V	C	LIN, TET, TMP
		t011-V (2)					
Pig/cattle 2	5/10 (pig)	t011-V (12)	pig (1)	t011	V	F	CIP, ERY, LIN, TET, TMP, TYL
	5/10 (cattle)		pig (1)	t011	V	F	CIP, TET, TMP
			cattle (1)	t011	V	E	CIP, TET, TMP
			cattle (1)	t011	V	E	TET, TMP
Pig/cattle 3	5/10 (pig)	t011-IVa (1)	pig (1)	t011	IVa	L	ERY, GEN, KAN, LIN, TET, TMP, TYL
	1/10 (cattle)	t011-V (5)	cattle (1)	t011	V	F	LIN, TET, TMP
Pig/cattle 4	5/10	t034-V (3)	pig (1)	t034	V	I	CHL, CIP, LIN, TET
		t011-IVa (2)	pig (1)	t011	IVa	N	CIP, GEN, KAN, LIN, TET, TMP, TOB
Pig/cattle 5	3/10	t011-V (3)	pig (2)	t011	V	C	ERY, LIN, TET, TMP, TYL
Pig/cattle 6	1/10 (cattle)	t011-IVa (1)	cattle (1)	t011	IVa	C	GEN, KAN, LIN, TET, TMP, TOB
Pig/cattle 7	5/10 (pig)	t011-V (10)	pig (1)	t011	V	C	ERY, LIN, TET, TMP, TYL
	5/10 (cattle)		pig (1), cattle (3)	t011	V	C	TET, TMP
			cattle (1)	t011	V	C	TET, TMP
			cattle (1)	t011	V	K	TET, TMP
Pig/cattle 8	4/10	t011-V (4)	pig (1)	t011	V	C	ERY, LIN, TET, TMP, TYL
			pig (1)	t011	V	N	ERY, LIN, TET, TMP, TYL
Pig/cattle 9	5/10 (pig)	t011-IVa (5)	pig (1)	t011	IVa	M	ERY, GEN, KAN, LIN, TET, TMP, TYL
	1/10 (cattle)	t011-V (1)	pig (1)	t011	IVa	B	ERY, GEN, KAN, LIN, TET, TMP, TOB, TYL
			cattle (1)	t011	V	E	LIN, TET, TMP

[a] When MRSA was isolated from poultry and cattle besides pigs, the species is indicated between brackets.
[b] Overview of the obtained SCC*mec*-*spa* type combinations of all obtained isolates
[c] Results of the selected isolates (n=62)
[d] When a non-typeable SCC*mec* type was obtained, the method of Kondo et al. (2007) was performed and the *mec* and *ccr* complex results are shown (NT: Non-typeable)

5 Discussion

Since the first report of MRSA ST398, many countries have reported on this MRSA type in livestock animals. In Belgium, multispecies farms (pig/poultry and pig/cattle farms) are regularly present. To our knowledge, this is the first report in which the MRSA presence in mixed farms has been studied to determine whether the different animal species have an influence on each other in terms of MRSA presence and genetic diversity of the obtained MRSA isolates.

The presence of MRSA, we observed in the different animal species within the farm types, is consistent with findings of other research groups (Graveland et al., 2008; Persoons et al., 2009; Dewaele et al., 2011; Pletinckx et al., 2011). During the present screening study, no statistically significant effect of the farm type was observed. In addition, there was no correlation between the number of positive cows or poultry and the number of positive pigs. From the current data, it is not possible to determine whether pigs have an influence on the MRSA status of poultry and cattle, because no poultry-only and cattle-only farms were sampled. We found that pigs were more likely to carry MRSA compared to cattle and poultry. Several factors might explain this difference of MRSA presence. First, the different animal species are physically separated from each other and have no direct contact, which reduces the opportunity for direct MRSA transmission. However, indirect transmission between the different animal types might occur through the farmer. Farmers are often nasal carriers of LA-MRSA and their clothing and footwear can become contaminated (Denis et al., 2009; Vanderhaeghen et al., 2010a). Farmers usually change footwear upon entering the poultry barn, which reduces the likelihood of transmission. Farmers also change shoes and/or clothes upon milking. Manipulation time differs among the species, however. On pig farms, farmers spent approximately 3-4 h in the nursing units and walked daily through the other pig units. On pig/cattle farms, dairy cattle were milked twice each day. Milking and walking through the barn resulted in a manipulation time of at least two to four hours each day. On pig/poultry farms, farmers walked through the poultry barns on daily basis and spent less than one hour in these barns. So, farmers have closer and longer-lasting contact with pigs and dairy cattle compared with poultry, which could result in the low and intermediate MRSA isolation rates in poultry and cattle, respectively. Second, poultry and cattle could be biologically less susceptible to acquire MRSA ST398 compared with pigs. However, this hypothesis should be confirmed by host-pathogen interaction studies. Third, during the present study, only ten

animals per animal species present were sampled, which might result in an underestimation of the within-herd prevalence of the MRSA colonization of the animals. Nevertheless, the present study gives an indication of the within-herd prevalence of the MRSA colonization in pigs, cattle and poultry.

Various methods were used for molecular typing of the isolates. MLST revealed the presence of ST398, which is associated worldwide with pigs. Moreover, in poultry and cattle, other STs such as ST9 and ST1 have been described which belong to a different CC than CC398 (Pilla et al., 2012; Szabó et al., 2012). Although only low numbers of isolates were retrieved from cattle (n=14) and poultry (n=1), no other CCs were found in these animal species during the present study. This finding suggests that other CCs have not yet spread in Belgian mixed farms, but additional samplings are needed to confirm this suggestion.

Spa typing is a typing method which rather reflects short-term evolution than long-term phylogenetic changes (Boye and Westh, 2011). In general, only one *spa* type per farm was seen. The presence of two *spa* types within one farm could be due to mutations in the strains present on the farm (e.g. deletion of one repeat in s*pa* type t011 results in *spa* type t1451 or t3423 and *vice versa*) or introduction through animal import, humans visiting the farm, pets, pests (such as rats and flies), etc (Van Duijkeren et al., 2008). In comparison with multispecies farms, more *spa* types were found on pig-only farms. When more discriminatory methods, such as PFGE and MLVA were used, a variety of genotypes was obtained (Rasschaert et al., 2009). For example, *spa* type t011 was associated with nine pulsotypes and seven MLVA types, resulting in 11 genotypes. This variety in genotypes within one *spa* type and the presence of approximately one *spa* type per farm indicates that the MRSA CC398 is a rather heterogeneous population of MRSA.

The observed antibiotic resistance rates to tetracyclin, trimethoprim, macrolides, lincosamides and aminoglycosides found on the different farm types were also reported by other research groups (Vanderhaeghen et al., 2010a). A remarkable difference between the farm types is the presence of ciprofloxacin resistance in pig isolates on five of eight pig/poultry farms compared with the presence of this resistance on only one pig-only farm and one pig/cattle farm. Differences in antimicrobial resistance types might be explained by the presence of different treatment methods for the different animal species or individual treatment of an animal favoring resistance to a specific agent. Another reason could be the presence of different additional resistance genes on plasmids in some MRSA isolates. Within a farm,

some isolates may lack certain genes or gene cluster, resulting in less antimicrobial resistance and thus a different profile (Kadlec et al., 2009). To better understand the resistance mechanisms of the isolates with different profiles, the presence of resistance genes should be studied. In the present study, the presence of gentamicin, kanamycin and tobramycin resistance appeared to be linked to the SCC*mec* IVa type. This was also seen by Gomez-Sanz et al. (2010) and Crombé et al. (2012a), but not by Vanderhaeghen et al. (2010b).

6 Conclusion

In this research, we have studied the MRSA presence in pig-only farms versus multispecies farms. We observed no correlation between the number of positive chickens/bovines and the number of positive pigs. Moreover, MRSA was isolated more often from pigs than from poultry and cattle. This difference might be explained by physical separation of the animals, the possibility of only indirect contact between the animals through the farmer or other (host-related) factors. Additional studies are needed to determine transmission routes on these farms and to gain insight into the effect of farm management on this transmission.

This study also associated a new *spa* type, t5943, with MRSA ST398. In general, *spa* and SCC*mec* typing revealed the presence of only one combination per farm. In addition to those techniques, other molecular typing methods should be used, because the use of PFGE and MLVA revealed a wide variety within the MRSA ST398 strains of certain farms. The significance of this genetic variety will require further research.

7 Acknowledgements

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Chapter IV. A. Cohort study for the presence of livestock-associated MRSA in piglets: effect of sow status at farrowing and determination of the piglet colonization age

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1 Abstract

A longitudinal study was performed to determine the age at which piglets become colonized with livestock-associated MRSA and the effect of the sow MRSA status on the colonization status of their offspring.

On four farrow-to-finish farms (A-D), nasal swabs were collected during a 6-month period from 12 sows and their offspring per farm. Piglets and sows were sampled throughout the nursery period. Additionally, the piglets were sampled after weaning, before and after moving to the finishing unit and before slaughterhouse transport. The environment of one pen (wall, floor and air) was sampled every time the pigs were sampled.

Two MRSA colonization profiles were observed. On farms A and B, the sows' colonization prevalence reached 17% and 33%, respectively. The proportion of positive piglets remained low in the nursing unit (farm A: 0-7%, farm B: 0-36%) and increased at the end of their stay in the growing unit (farm A: 91%, farm B: 69%). On farms C and D, the sows' and piglets' colonization percentages were high from the beginning of the sampling series and finally reached 100%. On all farms, a decrease in colonization was observed towards slaughter age. The colonization age differed between farms. Statistical analysis on the sampling results of farms A, B and C revealed a significant effect of the sow status at farrowing on the piglets' status.

The present study indicates that the sow's colonization status is important and should be included in control measures. However, the observed differences in colonization percentages among the farms complicate implementation of control measures on the farm.

2 Introduction

In the mid-2000s, a new methicillin-resistant *Staphylococcus aureus* (MRSA) type was described on pig farms in the Netherlands (Voss et al., 2005). Since then, research groups worldwide have reported on this pathogen, called "livestock-associated MRSA" (LA-MRSA). LA-MRSA has been isolated from various animal species such as pigs, cattle, chickens and horses and also from humans who come into close contact with these animals (Cuny et al., 2009; Van Duijkeren et al., 2010; Vanderhaeghen et al., 2010a; Pletinckx et al., 2011; Crombé et al., 2012a).

Many pigs are colonized with LA-MRSA but infections are rare, indicating that LA-MRSA can be considered a relatively minor animal health care problem (Van Duijkeren et al., 2007). However, colonized pigs may act as an MRSA reservoir for the general human population. LA-MRSA transmission from a farm to the general human population appears to be rare, but it is important to keep the potential risk of transmission low (Cuny et al., 2009; Smith and Pearson, 2011). Eliminating LA-MRSA from pigs will help to reduce the potential risk of LA-MRSA entering the human or animal population. One risk-reducing measure is to prevent colonization of the animals. Until birth, the piglets are located in the uterus, where no contact with MRSA is expected. During birth and further, pre-weaned piglets have contact with the environment and their mother which can result in LA-MRSA colonization (Weese et al., 2010a; Moodley et al., 2011a). Estimating the colonization age can help to implement control measures.

In the present study, a cohort study was performed on the offspring of 48 sows, originating from four farrow-to-finish farms. The aims of the study were (i) to determine the LA-MRSA colonization age of piglets, (ii) to gain insight into the possible effect of the sow colonization status on their offspring colonization status, (iii) to determine the effect of the environment and (iv) to determine the MRSA carriership of the piglets.

3 Materials and methods

3.1 Sample collection

Four Belgian pig farms, located in Flanders (the northern part of Belgium), were selected after having been screened for MRSA (Verhegghe et al., 2012). Inclusion factors for the farms were as follows: located in Flanders, being a farrow-to-finish farm and having MRSA positive

pigs (as determined from prior screening). Every farm was sampled during a 6-month period from July 2009 to December 2010. All farms were farrow-to-finish farms and operated in an all-in all-out manner in the nursing unit and growing unit. This type of management was also used on farm D, where the animals were reared in two finishing units and transported from finishing unit-1 to finishing unit-2 (see further). Farms A and B reared pigs only, whereas farms C and D reared pigs and broilers (approximately 50,000 broilers per farm). After finishing, pigs were directly transported to the slaughterhouse. Farm-specific characteristics, i.e., number of sows, production system, piglet age at weaning, etc., are shown in Table IV-1. Farm C is the only farm located on two sites. The distance between the two sites was approximately 30 km. After staying 1-2 weeks in the growing unit of location 1, the sampled group of piglets on farm C was transported to the second location. At this location, they resided approximately 5 weeks in the growing unit after which they were moved to the finishing unit.

The first 12 sows farrowed on the first sampling day were selected for the sampling events on farms A, B and C. On farm D, the sows were not sampled at farrowing because only one sow farrowed per day. On this farm, we sampled the animals every third day until 12 sows and litters were obtained. On farm A, all sows were located in one nursing unit. The 12 sows of farm B were spread over three nursing units. On farms C and D, they were spread over three and two units, respectively. On each farm, both nares of each sow and 10 piglets of their litter were sampled using pre-moistened nasal swabs. After sampling, the swabs were stored in salt-enriched (6.5% wt/vol; sodium chloride; 1.06404; Merk, Darmstadt, DE) Mueller Hinton Broth (MHB CM0405; Oxoid, Basingstoke, UK). At farrowing, the piglets were marked with a number and at the age of 5-7 days, they received a numbered tag. The number of piglets sampled per farm is given in Table IV-1. The decline in the number of sampled pigs/piglets was due to either tag loss or animal death. The sows were sampled within 2h after farrowing (except for farm D) and again together with the sampling of the pre-weaned piglets. When less than 10 piglets were present in one litter, more piglets were given to the sow from other litters. This was decided by the farmer when the sows had too little or too many piglets. Out of 12 sows on both farms A and B, seven and three sows, respectively, were given an additional piglet from another litter. On farm C, this applied to one sow, whereas on farm D, none of the sows were given an additional piglet. The piglets were sampled on several occasions. The timing of the sampling events, weaning and transport to the units are shown in Table IV-1. In short, in the nursing unit sampling occurred within an hour of being born (not

on farm D), on day 1 (not on farm D), day 3, day 5 (not on farm D), day 7 and before weaning. Samples were taken at the beginning and end of the piglets' stay in the growing unit. On farm C, where the animals were transported to the second location 8 days after weaning, one additional sampling event occurred 2 days after transport. On farm D, the animals were sampled every week in the growing unit. In the growing units of farms A and C (both locations), all piglets from the test group were mixed and divided into one and four pens (two barns), respectively. On farm B, the number of pens in the growing unit was equal to the number of pens in the nursing unit. The litters were kept together. On farm D, the sows were removed from their piglets and the nursing unit thus became the growing unit. Finally, the animals were sampled twice in the finishing unit (at the beginning of their stay in the finishing unit and at slaughter age). On farm A, the animals were spread over eight pens in one barn, whereas on farm C, the animals were spread over two barns with eight pens each. On farm B, the test group was located into two barns with four pens each. On farm D, the animals were reared in two finishing units (8 pens each). The pigs were moved to the second unit after 4 weeks in the first unit. Two additional sampling events occurred, one before moving to the second unit and the second upon entry into the second unit.

On every sampling occasion, one sample from the wall, the floor, the air surrounding the animals and the outgoing air was taken. From every unit/barn, as described above, one pen was sampled. 10 cm² of the walls and floors were sampled, using an envirosponge (3M Dry Sponge; Led Techno; BP133ES; St-Paul, MN, US) pre-moistened with 7 ml salt-enriched MHB and a sterilized frame. An air sampler (Air Sampler RCS; Biotest Hycon, Dreiech, DE) was used to sample 100 L of air during 2 min at shoulder height of the animals in one pen as well at the outgoing ventilation opening of the unit. The airstrips contained Oxacillin Resistance Screening Agar Base (ORSAB CM1008; Oxoid, Basingstoke; UK), which is a selective MRSA medium. All samples were transported directly to the laboratory and were processed immediately upon arrival.

3.2 Sample processing

The envirosponges were diluted 10 times with salt-enriched MHB. The swabs, air strips and sponge dilutions were incubated overnight (18-20h, 37°C). The next day, 1µl of the swabs and sponge dilutions and four blue colonies per airstrip were plated onto a chromogenic selective medium for MRSA (Chom-ID^{TM} MRSA; BioMerieux, Marcy l'Etoile, FR) and incubated overnight (18-20h, 37°C). One suspect colony was purified on Chrom-ID^{TM} MRSA

and after overnight incubation at 37°C was plated onto tryptone soy agar (TSA; CM0131; Oxoid, Basingstoke, UK). Pure isolates were stored at -20°C in brain-heart infusion broth (BHI; CM0225; Oxoid, Basingstoke, UK) supplemented with glycerol (15% wt/vol; Fisher Scientific, Leicestershire, UK) until further processing.

3.3 MRSA confirmation

The method of Strandén et al. (2003) was used to extract DNA of each isolate and these lysates were then stored at -20°C until further use. MRSA confirmation occurred through a multiplex PCR as described by Maes et al. (2002). To confirm the presence of LA-MRSA, *spa* typing was performed on 40 isolates per farm (arbitrarily selected sow and piglet isolates) according to the Ridom StaphType standard procedure (http://spaserver.ridom.de, Harmsen et al., 2003).

3.4 Statistical analysis

Only the sampling data from farms A, B and C were included because no results at farrowing were obtained from farm D. In total, 3510 records were imported to the analysis. A logistic mixed regression model was fit using MLwiN 2.1 (Rasbash et al., 2009) to test the effect of the farm and MRSA status of the sow at farrowing (independent variables) on the probability of a piglet to test positive for MRSA (dependent variable), from birth till transport to the slaughterhouse. In a first step, a three level model was tested in which the factors sow, piglet and time were included as random effects and number of measurement (categorical variable: <1 h; on day 1; 3; 5 and 7; before weaning; in the growing unit after weaning and before moving to the finishing unit; at the beginning of the finishing unit and before transport to the slaughterhouse) was forced into the model as fixed factor to correct for clustering of piglets within sows and for repeated measures within piglets. The random factor piglet was not significant. Consequently, piglet was no longer included as a random factor and a two level model was built. Second, univariable associations were tested between the independent variables, sow status at farrowing (0 = MRSA-negative and 1 = MRSA-positive) and farm as a fixed factor and the dependent variable being MRSA status of the piglets. Difference was considered significant when $P < 0.05$. The proportion of the variation for MSRA status of the piglets at sow and time level were estimated by assuming that the variance at time level on the logit scale was $\pi^2/3$ (Dohoo et al., 2001).

Table IV-1 Overview of various characteristics of the four sampled farrow-to-finish farms (h: hour, Fu1: finishing unit 1, Fu2: finishing unit 2).

	Farm			
	A	B	C	D
Number of sows	240	200	300	180
Livestock species present	Pigs	Pigs	Pig-poultry	Pig-poultry
Production system (weeks)	3	3	4	1
Piglet age at weaning	27	24	20	24
Transport to finishing unit	69	66	74	Fu1:69, Fu2:120[a]
Sampling events (days):[b]				
Nursing unit	<1h/1/3/5/7/17	<1h/1/3/5/7/23	<1h/1/3/5/7/17	3/7/23
Growing unit	28/52	30/58	21/33/54[c]	31/39/45/52/59/67/82
Finishing unit	75/187	67/165	88/172	Fu1:90,117, Fu2:123/174[a]
Number of:				
Breeding/gestation unit	1	1	1	2
Nursing units	2	7	6	10[d]
Growing units	12	9	2 (L1), 4 (L2)[e]	10[d]
Finishing units	7	12	4 (L1), 5 (L2)[e]	16[f]
Nursing units with sampled sows	1	3	5	2
Number of:				
Sampled piglets at farrowing	111	125	115	120
Sampled pigs at slaughter age[g]	94	94	82	99
Antibiotic treatment in growing unit	Yes[h]	Yes[h]	Yes[h]	Yes[h]
Cleaning measures				
Cleaning water	Cold (15°C)	Cold (15°C)	Tepid (25-30°C)	Tepid (25-30°C)
Disinfection	No	No	Yes	Yes
Fixed route[i]	Yes	Yes	No	No
Distance to other pig farms	>3km	>1km	<100m	<1km

[a] The test group on farm D resided in two finishing units, [b] The sampling events are shown in days after farrowing, [c] The test group was transported to the second location on day 31 and was sampled twice there, [d] Nursing unit becomes growing unit after weaning, [e] L1: location 1, L2: location 2, [f] Eight finishing units ("unit 1"), where the animals resided 5 weeks after the growing unit and eight finishing units ("unit 2"), where the animals resided until slaughter age, [g] Decrease in number is due to death or tag loss, [h] Farms A and C: promycin and amoxicillin, farm B: trimethoprim and sulfadiazine and farm D: amoxicillin, [i] Farmer always walks through the farm according to a fixed route

The MRSA colonization age was defined as the age of a piglet on which MRSA was detected for the first time. We determined this age per farm and calculated the average colonization age from farms A, B and C.

3.5 Results

Table IV-2 represents the results of the sow sampling events. In short, on farms A and B, MRSA was isolated only sporadically from the sows, whereas on farms C and D, the majority of the sows in the nursing unit were carrying MRSA.

Table IV-2 Overview of the number and percentage of MRSA positive sows per farm (12 sows sampled per farm) and per sampling event in the nursing unit. The pre-weaning sampling event occurred on day 17 on farms A and C and day 23 on farms C and D.

MRSA positive sows/sampling event (n=12)								
	Farm							
Time after farrowing	A		B		C		D	
	n	%	n	%	n	%	n	%
<1 h	0/12	0	0/12	0	6/12	50	NA	NA
Day 1	1/12	8	1/12	8	10/12	83	NA	NA
Day 3	0/12	0	0/12	0	10/12	83	11/12	92
Day 5	2/12	17	4/12	33	12/12	100	NA	NA
Day 7	1/12	8	0/12	0	12/12	100	12/12	100
Pre-weaning	0/12	0	0/12	0	10/12	83	12/12	100

[a] NA: not available

The sampling results in the successive rearing stages of their piglets on the four farms are shown in Figure IV-1. On farms A and B, low prevalences were observed in the piglets until the end of their stay in the growing unit, when the prevalence increased. On farms C and D, the piglet prevalences remained high throughout their lifespan. On all farms, the prevalence declined at slaughter age.

None of the piglets on farms A and B carried MRSA at every sampling event, whereas on farms C and D, 46% and 39% of the piglets carried MRSA at every sampling event, respectively. On farm A, 1% of the piglets remained MRSA-free throughout the whole study. Moreover, on farm A, MRSA was isolated only once from 12% of the piglets during all sampling events. In the remaining piglets, MRSA was detected twice or more. On farm A, 23% of the animals tested MRSA negative at least once between two positive samples. On farm B, 5% of the piglets were found to be colonized with MRSA only once. The remaining piglets tested positive twice or more. On farm B, 69% of the animals tested MRSA negative at least once between two positive samples. On farm C, 12% of the piglets were MRSA negative at least once between two positive samples. On farm D, this occurred in 43% of the animals.

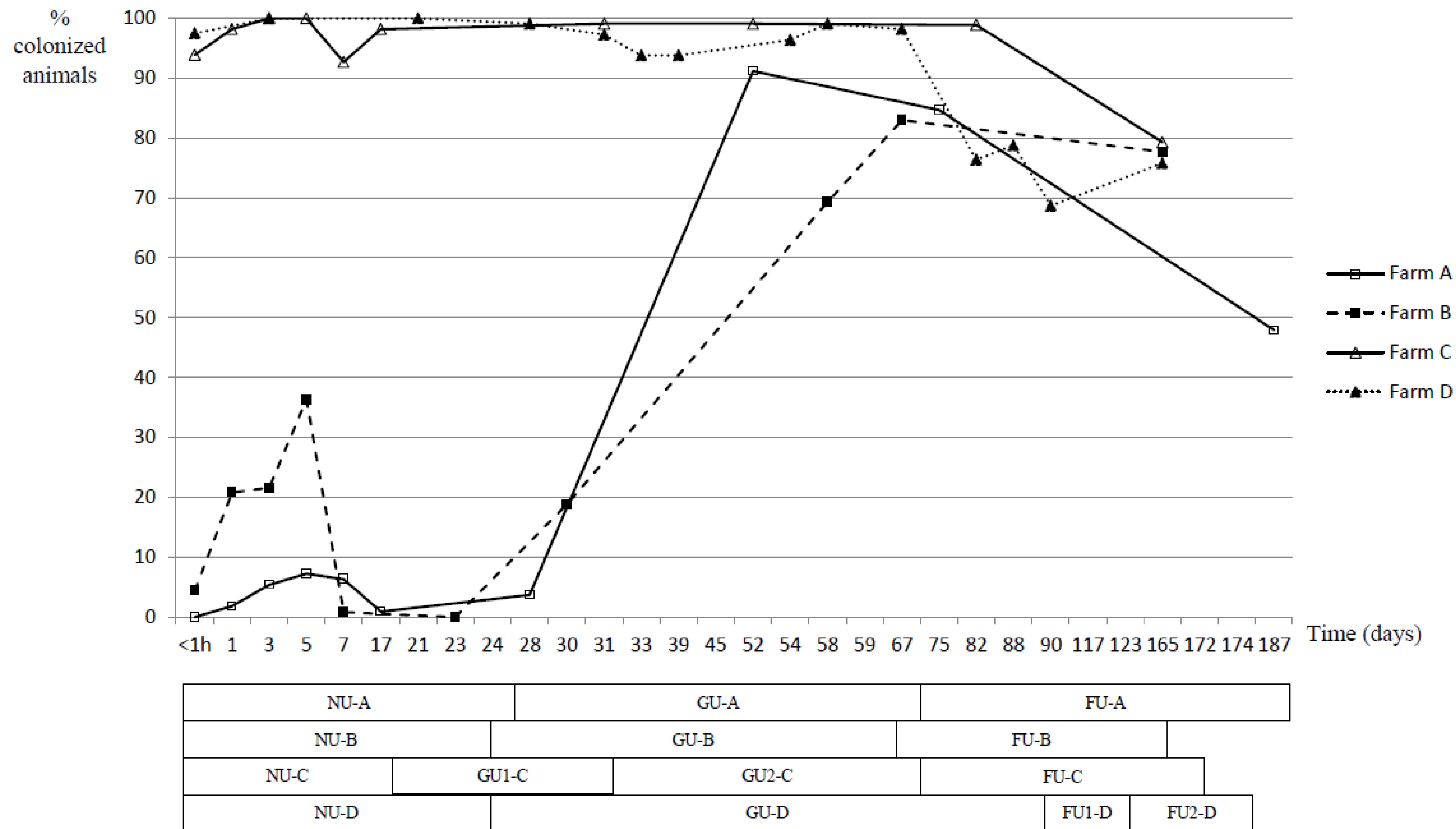

Figure IV-1 Percentage of colonized piglets at the four farms taken in the different rearing units at different time points (h: hour, NU: nursing unit, GU: growing unit, GU-1: growing unit 1, GU-2: growing unit 2, FU: finishing unit, FU-1: finishing unit 1 and FU-2: finishing unit 2).

The average MRSA colonization age of the piglets was 17.8 days [95% CI: 15.3-20.2], which ranged from 46.6 days [95% CI: 41.3-52.1] on farm A to 24.0 days [95% CI: 18.7-29.3] and 0.1 days [95% CI: 0.22-0.47] on farms B and C, respectively. On farm D, no samples were taken at farrowing, thus no colonization age could be determined.

In the nursing units of farms A and B, MRSA detection remained low in the environment. In the growing and finishing unit of both farms, MRSA was detected on the floors and walls. MRSA isolation occurred only in the air of the finishing units. On farms C and D, MRSA was isolated during all sampling events from the floors, walls and air (data not shown).

The variance components of the factors sow and time, when included randomly in the model, were 2.48 and 3.29, respectively. The results of the univariable and multivariable models are shown in Table IV-.3. From these univariable models, it was shown that time, sow status at farrowing and farm have a significant effect on the MRSA status of the piglet. When farm was included as a fixed factor in the multivariable model, none of the other independent variables were significant. Because farm related factors such as management were not the focus of this article, a multilevel model with a factor other than farm was also investigated (Table IV-3). In this multilevel model, time, sow status and the interaction between time and sow status were found to have a significant influence on MSRA status of the piglet. Figure IV-2 shows a prediction of the probability for a piglet to contract MRSA when originating from a MRSA positive or negative sow. A piglet originating from a MRSA negative sow has a lower statistically significant probability to become MRSA colonized than a piglet originating from a MRSA-positive sow until time point 7. The probability started to increase from time point 8 on, where no significant differences in probabilities between piglets of both groups were observed. Because the interaction between sow status and time was significant, stratum specific odds ratio's were calculated and plotted (Figure IV-3).

Spa typing revealed the presence of only *spa* type t567 on farm A, whereas only type t011 was found on the three other farms.

Table IV-3 Results of the statistical analysis of the univariable (UV) and multivariable (MV) models.

Model	Factor	β	SE	OR	95% CI	p
UV	Time					<0.001
	Sow status					<0.001
	0	Ref				
	1	2.549	0.712	12.79	3.17-51.65	
UV	Time					<0.001
	Farm					<0.001
	A	Ref				
	B	0.732	0.215	2.08	1.36-3.17	
	C	5.747	0.279	313.25	181.30-541.23	
MV	Cte	-1.514	0.349			
	Time					<0.001
	1	Ref				
	2	0.560	0.205	1.75	1.17-2.62	
	3	0.711	0.203	2.04	1.37-3.03	
	4	1.041	0.199	2.83	1.92-4.18	
	5	0.319	0.211	1.38	0.91-2.08	
	6	0.147	0.217	1.16	0.76-1.77	
	7	0.640	0.206	1.90	1.27-2.84	
	8	3.128	0.229	22.83	14.57-35.76	
	9	3.474	0.249	32.27	19.81-52.56	
	10	2.173	0.211	8.78	5.81-13.28	
	Sow status					<0.001
	0	Ref				
	1	2.396	0.773	10.98	2.41-49.95	
	Sow status 1xTime					0.01
	1-2	0.260	0.484	1.30	0.50-3.35	
	1-3	0.233	0.494	1.26	0.48-3.32	
	1-4	0.039	0.508	1.04	0.38-2.81	
	1-5	0.502	0.490	1.65	0.63-4.32	
	1-6	0.673	0.493	1.96	0.75-5.15	
	1-7	0.303	0.502	1.35	0.51-3.62	
	1-8	-0.556	0.819	0.57	0.12-2.86	
	1-9	-1.452	0.707	0.23	0.06-0.94	
	1-10	-1.309	0.537	0.27	0.09-0.77	

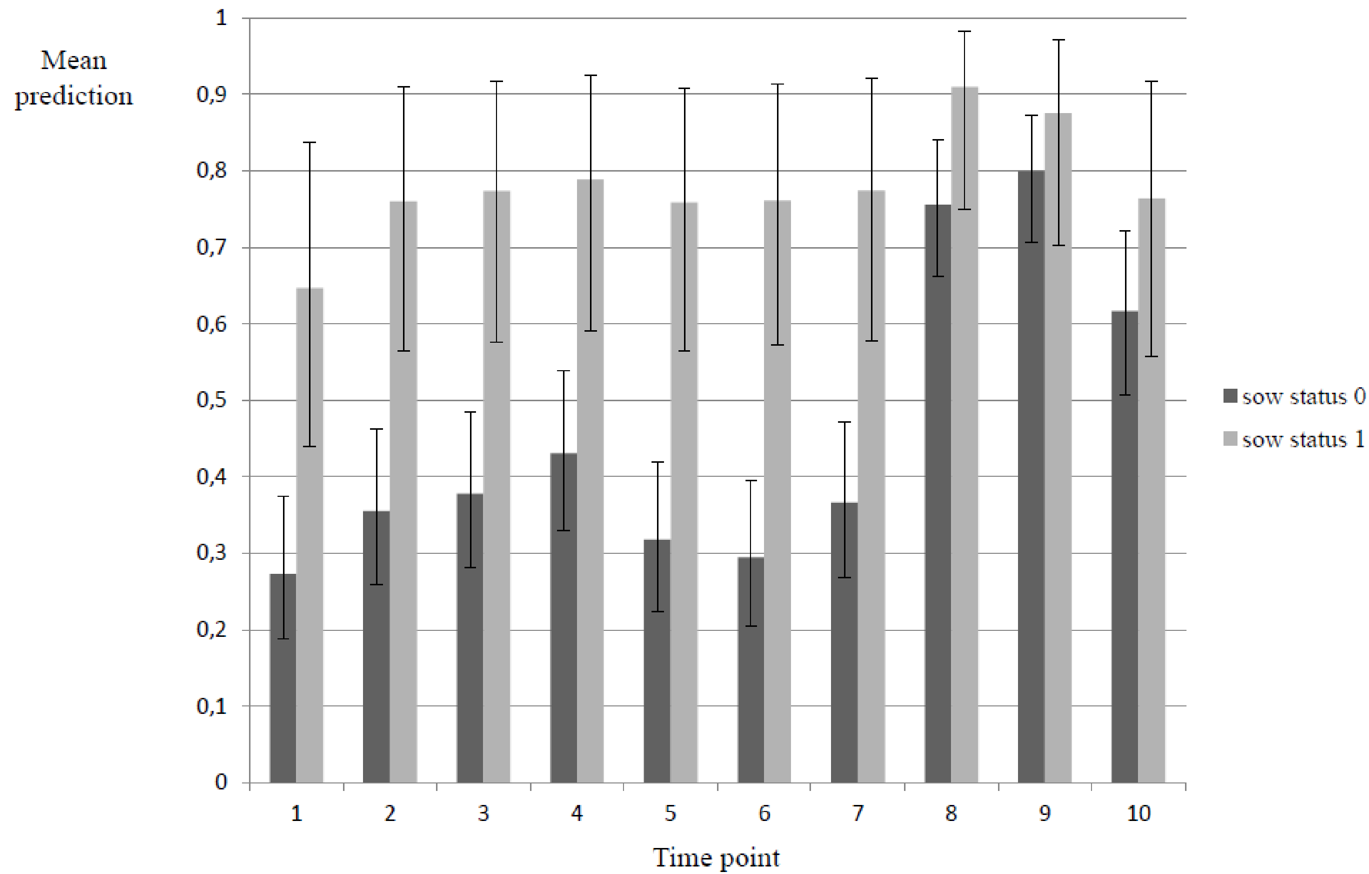

Figure IV-2 Prediction of the probability of a piglet to contract MRSA at a certain time point when originating from an MRSA-positive (status 1) and MRSA-negative sow (status 0). Time points 1-6 represent the sampling events in the nursing unit within 1 h, 1 day, 3 days, 5 days, 7 days after farrowing and before weaning. Time points 7 and 8 represent the sampling events in the growing unit; points 9 and 10 represent the sampling events in the finishing unit.

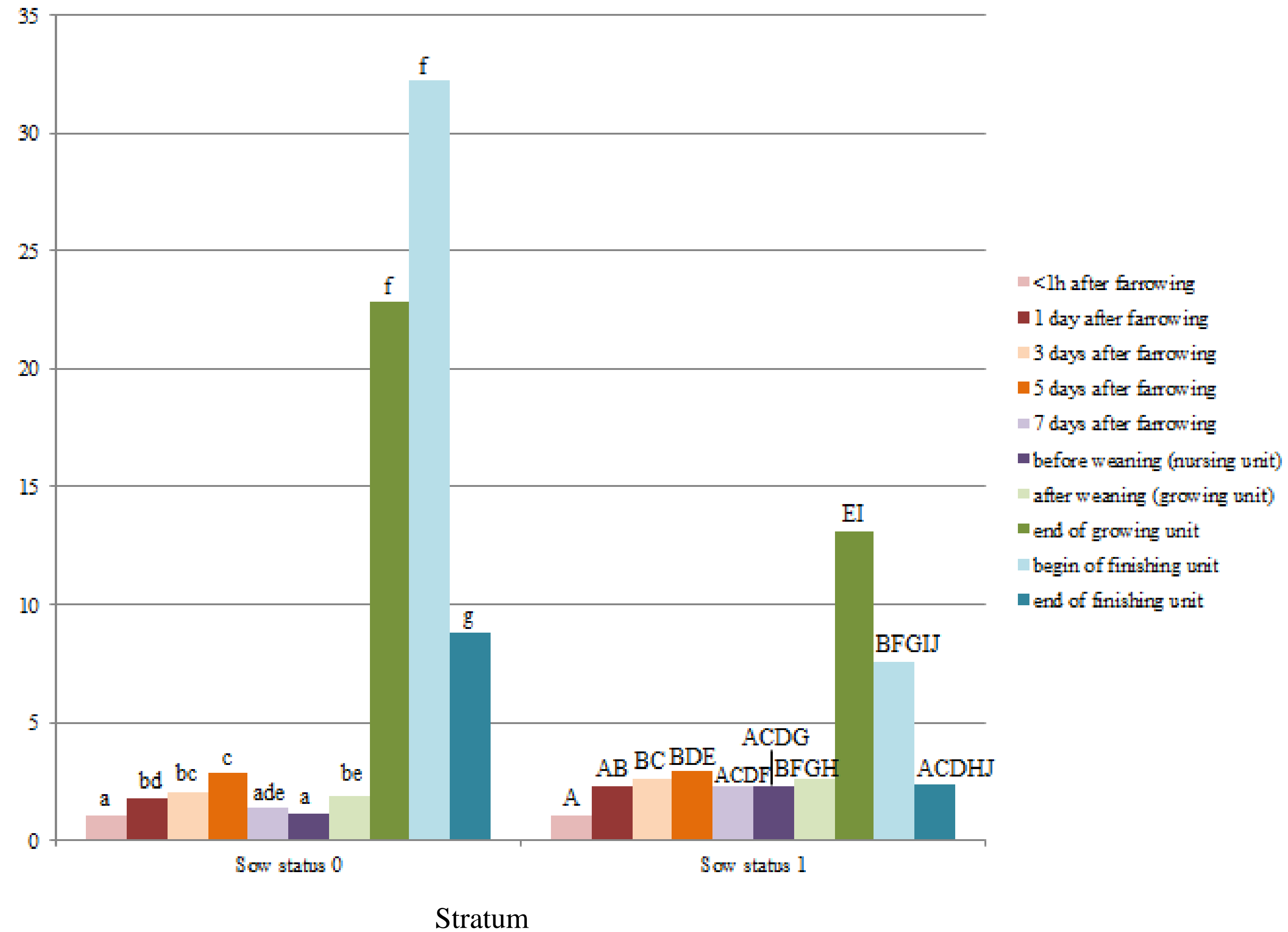

Figure IV-3 The odds ratio for a piglet to retrieve MRSA at a certain time point when originating from a MRSA-negative sow (=stratum 0) and a MRSA-positive sow (=stratum 1). The different letters indicate significant differences within one stratum.

4 Discussion

Ever since the first description of LA-MRSA in pigs, concerns have arisen about the transmission of this pathogen to the human population. To decrease this transmission probability, it is important to reduce the amount of MRSA present on the farm. Before control measures can be implemented, information is needed about the factors that might influence MRSA dynamics within a farm. To gain insight into MRSA carriage over time and the sow-piglet dynamic, a cohort study was performed on four farrow-to-finish farms.

One difficulty that was encountered during the present study was defining colonization and contamination of the animals. Pirofsky and Casadevall (2002) suggested that colonization was a state whereby a potential microbe is recovered from a non-sterile site without evidence that the microbe is causing disease. During the present study, it was assumed that an animal was colonized with MRSA when MRSA was isolated from the nares. The present study and, to our knowledge, other studies do not provide enough evidence for an accurate definition of colonization and contamination.

When observing the colonization percentages of sows and piglets, two types of farms were observed. Farms A and B were defined as low colonization farms, whereas farms C and D can be defined as high colonization farms. When comparing the farms, some operational dissimilarities were observed. A first notable difference between the farms was the presence of poultry on farms C and D. Second, no other pig farms were located in the proximity (1 km) of farms A and B in contrast to farms C and D. Third, farms A and B both applied a three-week production cycle, whereas farms C and D used a 4-week and 1-week cycle, respectively. Furthermore, on farms A and B, the barns were cleaned with cold water without disinfection, whereas on farms C and D tepid water and a disinfection step were used. Last, the farmers on farms A and B used a fixed walking route through the farm i.e. first the nursing and breeding units and then the other parts of the farm, which was not used on farms C and D. A thorough epidemiological study is needed to determine which factors are of importance for the observed MRSA colonization trends.

In the present study, four separate piglet populations were sampled. Statistical analysis revealed a statistically significant effect of the farm. This was expected; comparison of the sampling data of the different farms demonstrated a large variation. Furthermore, a farm can be considered as a closed system in which different factors might play a role in the

colonization of the animals. The mother sows are an important MRSA source for piglets, as they transmit MRSA through contact with the nose, skin, vagina, etc. (Moodley et al., 2011a). The MRSA status of the mother sow at farrowing had a significant influence on the piglets' MRSA status, which concurs with the report of Weese et al. (2010a). When a piglet originated from a MRSA positive sow, the probability for this piglet to contract MRSA at farrowing was higher than a piglet originating from a MRSA negative sow. The odds ratio's were determined for piglets originating from both sow types. When originating from a MRSA negative sow, the odds for a piglet to contract MRSA increased around the age of 9 weeks (time point 8). After weaning, no effect of the mother sow was anticipated, but other factors, such as antibiotics management, commingling of the animals and transport throughout the farm might have an additional effect on the odds of a piglet for retrieving MRSA. Still, additional research is needed to determine the influence of these factors.

In the present study, MRSA was frequently isolated from the floors and walls in relation with high MRSA detection in the piglets. It has been reported that the environment plays an important role in the transmission of MRSA (Smith et al., 2010). Contamination from the environment might be a possible explanation for the present results. However, more MRSA positive animals could increase the level of MRSA contamination in the environment, which would result in increased detection of MRSA. Both explanations still need confirmation in future studies as the present study provided insufficient data to make an accurate assessment.

On occasion, MRSA was isolated from piglets born of a continually MRSA-negative mother sow. This might be due to the detection limit of the sampling method, which could have resulted in false negative samples. Another possibility is the presence of intermittent MRSA carriers. In most piglets and some sows, no MRSA was detected at least once during the sampling period, which might be an indication of intermittent MRSA carriership or recolonization of the animals (Broens et al., 2011a; Crombé et al., 2012b).

A remarkable difference between the high and low colonization farms were the piglets' colonization percentages in the nursing and growing unit. Weese et al. (2010a) also reported an increase in colonization around day 42. In the study of Nathaus et al. (2010), an increase in colonization numbers was reported on one farm around day 21. On the low colonization farms, the colonization number only increased during the stay of approximately 4 weeks in the growing unit. As mentioned by Weese et al. (2010a), different factors might explain this increase, such as stress caused by weaning, commingling of MRSA-positive and negative

piglets, age related susceptibility and contact with contaminated farm sites. On all the farms in this study, piglets receive antibiotics upon entering the growing unit. This may cause a selection for resistant bacteria and lead to a detectable MRSA colonization (Broens et al., 2011a).

The last sampling event of the present study occurred at slaughter age. Remarkably, on all farms, the prevalence of MRSA was observed to drop near slaughter age. This decrease might be explained by age-dependent colonization, which has been observed by other research groups (Crombé et al., 2012a; Dewaele et al., 2011; Vanderhaeghen et al., 2010a). A comparison of the MRSA prevalence rates of pigs at slaughterhouses revealed that the colonization percentages found in these Flemish farms was within the range of the rates (30-70%) found in other high-density farming countries such as The Netherlands, Spain or Germany (de Neeling et al., 2007; Tenhagen et al., 2009; Gomez-Sanz et al,. 2010).

5 Conclusion

During the present study, two MRSA colonization trends were observed. These trends resulted in different colonization ages of the piglets. Sampling additional farms may confirm these observed trends and results or may reveal other trends. A statistically significant effect from the sow status at farrowing on the piglet MRSA status was observed.

The environment was also sampled. The present study could not confirm whether the environment contaminated the piglets or *vice versa*. To identify additional sources within a farm, additional samples should be taken on the farm and an epidemiological study should be performed. Moreover, piglets either appeared to be intermittent carriers or underwent recolonization over time. Molecular typing of the isolates might help to elucidate what is actually happening, together with potential MRSA sources, possible transmission routes, etc. Once the different sources are known, hygienic measures should be created or adapted to reduce the MRSA load on a farm.

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Chapter IV. B. Genetic diversity of livestock-associated MRSA isolates obtained from piglets from farrowing until slaughter age on four farrow-to-finish farms

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1 Abstract

During a previous longitudinal study, performed on four farrow-to-finish farms (A to D), samples were taken from twelve sows, their offspring and the environment on various occasions during six months. During the present study, a selection of the obtained MRSA isolates were typed by multiple-locus variable-number tandem-repeat analysis (MLVA), Pulsed Field Gel Electrophoresis (PFGE), *spa* typing and SCC*mec* typing to study the genetic diversity of LA-MRSA isolates and to determine possible MRSA sources for pig(let)s.

PFGE, *spa* typing and SCC*mec* typing revealed the presence of one or few dominant genotype(s) per farm. In contrast, 224 MLVA types were detected on the four farms, clustered together in one cluster on farms A and B, four on farm C and two on farm D. The genotype, found on farm A was unique for this farm. Farms B, C and D shared one cluster. In general, MLVA types from these clusters were isolated from piglets, sows and the environment on various sampling events. Piglets carried MLVA types both related and unrelated to their mother sows MLVA types at farrowing and onwards.

In conclusion, molecular typing revealed that within a farm one or a few dominant strain(s) are widespread. Potential MRSA sources for piglets were mother sows, the environment and other piglets.

2 Introduction

Since Voss and colleagues (2005) first described a new methicillin-resistant *Staphylococcus aureus* (MRSA) type in a pig farmer and his pigs, this livestock-associated MRSA (LA-MRSA) has been isolated from different livestock animals, especially pigs and from humans having close contact with them (Vanderhaeghen et al., 2010a; Weese, 2010). LA-MRSA rarely causes infections in pigs. Pigs are regarded as a potential source of MRSA for the human population, although at present, the risk of transmission appears low (Cuny et al., 2009; Smith and Pearson, 2011). To prevent dissemination of LA-MRSA in animals and humans, the MRSA load on pig farms should be reduced or eliminated. Implementation of, for example, hygienic measures might be useful but before implementing such measures, the main MRSA sources on a farm should be identified.

Molecular typing is very useful to investigate sources and vectors of pathogens. At present, different typing methods are available to study the spread of MRSA strains. Methods, such as multilocus sequence typing (MLST) and *spa* typing indicated that LA-MRSA, which is mostly MRSA ST398, is a rather clonal type with a limited set of *spa* types (Weese et al., 2010a). SCC*mec* cassette types IVa and V were mainly identified in these isolates. MRSA ST398 appeared non-typeable when using the gold standard Pulsed Field Gel Electrophoresis (PFGE) protocol with *Sma*I restriction which is due to a methylation of its restriction site (Bens et al., 2006). At present, other restriction enzymes such as *BstZ*I, *Apa*I and *Cfr*9I have been used instead (Rasschaert et al., 2009; Bosch et al., 2010). A more recent method to discriminate between clonal isolates is multiple-locus variable-number tandem-repeat analysis (MLVA). This method has been used for epidemiological studies of human *S. aureus* isolates or *Salmonella* isolates. MLVA is more discriminatory than MLST and *spa* typing and detects short-term evolution within strains (Rasschaert et al., 2009). This could be a good method in a pig farm setting where other typing techniques show too little variation between isolates.

In the present study, MLVA typing was used for the first time on a large subset of LA-MRSA isolates, obtained from a previous longitudinal study on four farrow-to-finish farms (Verhegghe et al., 2013a). Besides MLVA, the more classical typing methods (*spa* typing, SCC*mec* typing and PFGE) were used as well. The main goal of the present study was to investigate the genetic diversity of LA-MRSA isolates from sows, their piglets and their environment from farrowing till slaughter age to gain insight into this diversity on pig farms and to identify potential MRSA sources on the basis of genetic relationships.

3 Material and Methods

3.1 Isolate collection

From July 2009 to December 2010, four farrow-to-finish farms (A to D) were sampled during a six-month period (Verhegghe et al., 2013a). In short, on each farm, nasal swabs were collected from 12 sows and their offspring. From farrowing until weaning, the sows were sampled in the nursing unit on six occasions on farms A, B and C and on three occasions on farm D. Sampling of the piglets occurred from farrowing until slaughter age on 10 (farms A and B) and 11 (farms C and D) time points. On every sampling day, environmental samples were also taken from the wall, floor and air of one pen per stable. Farm C was located on two sites: piglets were born on site 1 where they resided until they were approximately five weeks old after which they were transported to the second site where they stayed until slaughter age. Bacteriological analysis of samples resulted in two trends according to Verhegghe et al. (2013a). Farms A and B were defined as low colonization farms, whereas farms C and D as high colonization farms. On the low colonization farms, MRSA was isolated sporadically from the sows and piglets in the nursing unit. The colonization percentage of the piglets increased at the end of the stay in the growing unit and remained high till slaughter age. On the high colonization farms, the colonization percentage of the sows and piglets in the nursing unit was high and remained high throughout the sampling events. In total, 3450 isolates were collected on the four farms: 262 and 407 isolates on farms A and B, respectively, and 1284 and 1497 isolates on farms C and D, respectively.

Due to this large number of obtained isolates, a selection was made and a total of 964 isolates were genetically characterized. From each farm, all sow isolates (A: n=4, B: n=3, C: n=45 and D: n=22) were included. Selection of the piglet isolates was different for the low and high colonization farms. On the low colonization farms (A and B), few MRSA was isolated from the piglets throughout the six-month period. From each sow, a variable number of piglets was selected, being piglets with the highest MRSA isolation rate in the litter. In total, 44 piglets of farm A and 45 piglets of farms B were chosen, resulting in 127 and 143 isolates, respectively. On the high colonization farms (C and D), MRSA was isolated from most piglets on all sampling events. To select these piglet isolates of these herds, eight and nine sows out of the twelve sows of farms C and D, respectively, were arbitrarily chosen. All isolates from three to four piglets of the selected sows were typed (n=278 on farm C, n=276 on farm D). In

addition, from the environmental isolates, all wall isolates were typed (A: n=4, B: n=9, C: n=27 and D: n=28).

3.2 Molecular typing

On all 964 isolates, multiple-locus variable-number tandem-repeat analysis (MLVA) was performed with a modified protocol of the method of Rasschaert et al. (2009). The repeat regions of five genes were amplified, being *clf*A, *clf*B, *sdr*C, *sdr*E and SIRU21. Fragment sizing of the PCR products was done by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems/Hitachi, Hitachinaka-shi, JP) using the Genescan™ 1200LIZ® size standard (4379950, Applied Biosystems, Warrington, UK). In each run, a MRSA ST398 strain (MV-162) was added as positive control. The obtained patterns were transformed into numeric codes using the MLVA plugin in Bionumerics (Bionumerics version 6.5; Applied Maths, St.-Martens-Latem, BE). Categorical analysis using the unweighted pair cluster method using averages (UPGMA) was performed (tolerance: 0%) to obtain the MLVA types per farm. For convenience and clear representation of the results, each MLVA numeric code (a string of five integers) was converted to an MLVA type with a unique number (for example: MLVA numeric code 32-46-38-38-2 of farm A was converted into MLVA type 11). In addition, a Minimum Spanning Tree (MST), based on the numeric code, was generated per farm in Bionumerics 6.5. Due to the large variety of MLVA types per farm, clustering of these types was performed. Each cluster consisted of a dominant MLVA type with closely related types, being single locus variants (= MLVA types with one difference in one repeat region). For example, the predominant MLVA type 11 (32-46-38-38-2) of farm A was closely related to eight other MLVA types, belonging to cluster A. The clusters were indicated on the MST of each farm. MLVA types containing only one isolate were defined as singletons.

Pulsed Field Gel Electrophoresis (PFGE) with *BstZ*I restriction (Promega, Madison, WI, US), as described by Rasschaert et al. (2009), was performed on 226 isolates in total: 41 of farm A (4 sow, 34 piglet and 3 environmental isolates), 43 of farm B (4 sow, 31 piglet and 4 environmental isolates), 86 of farm C (16 sow, 59 piglet and 11 environmental isolates) and 56 of farm D (eight sow and 48 piglet isolates). As a basis for this selection, we selected the sows arbitrarily to collect isolates belonging to various MLVA types. From each of these sows, one or two piglets were chosen with as many isolates (of various time points) as possible. In addition, the latter isolates belonged to as many MLVA types as possible. The obtained restriction profiles were analyzed using the unweighted pair cluster method using

averages (UPGMA) with the Dice coefficient (tolerance 1%, tolerance change 1% and optimization 1%). Pulsotypes were determined based on a delineation level of 97% (Bionumerics version 6.5; Applied Maths, St.-Martens-Latem, BE). The pulsotypes of each farm were given the farm letter accompanied with a roman number.

Spa typing and SCC*mec* typing was performed on 11, 11, 25 and 24 isolates of farms A through D, respectively. These isolates were arbitrarily chosen from every obtained pulsotype. The Ridom StaphType standard procedure (www.ridom.de/staphtype) was used for *spa* typing and the *spa* type was determined using the Ridom StaphType software (Ridom GmbH, Würzburg, DE). Three different protocols were used for SCC*mec* typing (Oliveira and de Lencastre, 2002; Zhang et al., 2005; Milheiriço et al., 2007) and results were combined to obtain the SCC*mec* type. When a non-typeable SCC*mec* type was found, the method of Kondo et al. (2007) was used and the *mec* and *ccr* complex is given. As a control, seven strains were used, carrying SCC*mec* cassette I, II, III, IVa, IVb, IVc and V.

4 Results

The MLVA method as described by Rasschaert and coworkers (2009) was changed during the present study: fluorescent primers were used to allow capillary electrophoresis and during the analysis in Bionumerics, a tolerance of 0% was used in contrast to the described tolerance of 1% (Rasschaert et al., 2009). This adapted protocol needed validation. During each run, the same MRSA sample was used and we observed a good inter and intra-run repeatability (data not shown).

4.1 Genetic diversity on the four farms

In total, 224 MLVA types were detected in the 964 isolates, originating from the different farms. Each MLVA type consisted of a five-string numeric code and received a unique number (Supplementary table S1). Closely related MLVA types (single locus variants = MLVA types differing in one repeat region) were clustered: for example, the predominant MLVA type 11 (32-46-38-38-2) of farm A was closely related to eight other MLVA types, belonging to cluster A (Figure IV-4, Table IV-4).

On farm A, 24 types were detected with MLVA of which 9 were clustered in one predominant cluster A (Table IV-4, Figure IV-4). *Spa* type t567 was detected in combination with a non-typeable (NT) SCC*mec* cassette type (*mec*A complex NT/*ccr* complex C). In addition, one predominant pulsotype was found on this farm (A-I, 98% of the tested isolates) (Figure IV-9). Fifty-seven MLVA types were observed on farm B, from which 16 were clustered in the predominant cluster B. Clusters F and G each contained four and two MLVA types, respectively (Table IV-4 and Figure IV-5). One pulsotype, *spa* type t011 and SCC*mec* type V was present in the isolates of this farm (Figure IV-9). The tested isolates of farm C belonged to 94 MLVA types (Figure IV-6A). Four predominant MLVA clusters (B, C, D and E) were present, each consisting of 5, 15, 4 and 3 MLVA types, respectively (Table IV-4). *Spa* type t011 was detected and the isolates carried SCC*mec* type IV (45%) or V (55%). Five pulsotypes (C-I, one predominant in 92% of the tested isolates) were present on farm C (Figure IV-9). On farm D, 49 MLVA types, of which 12 and 6 were clustered in MLVA clusters B and D, respectively, were present (Table IV-4 and Figure IV-7). *Spa* type t011 and SCC*mec* type V were observed together with two pulsotypes (D-I, one predominant in 93% of the tested isolates) (Figure IV-9).

Table IV-4 Overview of the MLVA clusters, being related MLVA types clustered together, present per farm. The dominant MLVA type or types per cluster are indicated. Clusters containing less than 10 isolates are clustered as other clusters. The remaining MLVA types were classified under not-clustered MLVA types.

MLVA cluster	Dominant MLVA type(s)[a]	Farm A		Farm B		Farm C		Farm D	
		# MLVA types in cluster	# isolates/ total	# MLVA types in cluster	# isolates/ total	# MLVA types in cluster	# isolates/ total	# MLVA types in cluster	# isolates/ total
A	32-46-38-38-2	9	113/135	-	-	-	-	-	-
B	33-57-37-7-3 33-57-37-6-3	-[c]	-	16	89/155	5	66/355	12	157/326
C	34-57-34-7-3	-	-	-	-	15	105/355	-	-
D	34-59-38-8-4	-	-	-	-	4	57/355	6	131/326
E	35-59-35-8-4	-	-	-	-	4	54/355	-	-
F	32-55-35-5-3	-	-	4	12/155	-	-	-	-
G	33-57-36-7-3	-	-	2	11/155	-	-	-	-
Other clusters[b]		8	14/135	11	11/155	18	20/355	9	12/326
Non-clustered		7	8/135	24	32/155	48	53/355	22	26/326

[a] VNTR code of the repeat region of the 5 genes clfA, clfB, sdrC, sdrE and SIRU21
[b] The number of other clusters is 3, 5, 9 and 4 for farms A-D, respectively
[c] -: not detected on the farm

Both SCC*mec* cassette types, present on farm C, were found in the isolates of one animal, as shown in the example in Table IV-5. SCC*mec* type IV was present in MLVA clusters B and D whereas type V was present in MLVA clusters C and E. Both cassette types were found in the predominant pulsotype C-I (Table IV-5).

Table IV-5 Molecular typing results of two sows (1 and 9) and two piglets of these sows (6 and 88) of farm C. All isolates belonged to *spa* type t011. The origin, sampling event, SCC*mec* cassette type, the MLVA numeric code, MLVA cluster or MVA type number in case of a non-clustered MLVA type and pulsotype is given.

Origin	Sampling event(s) (days after farrowing)	SCC*mec* cassette type	MLVA numeric code[a]	MLVA cluster or type	Pulsotype
sow 1	d3	V	33-57-37-6-3	B	C-I
	d17	V	33-57-36-6-3	1	C-I
pig 6	d1, d3, d54, d88	V	33-57-37-7-3	B	C-I
	d3	V	33-57-36-7-3	B	C-I
	d5, d17, d33	V	34-59-38-8-4	D	C-I
	d7	V	31-53-35-6-3	152	C-I
pig 88	d33	V	33-57-37-7-3	B	C-I
	d54, d88	V	34-59-38-8-4	D	C-I
	d172	V	26-59-38-8-4	139	C-I
sow 1	d1	IV	33-55-35-1-3	127	C-I
	d5	IV	33-53-34-8-4	171	C-I
pig 6	d21	IV	34-57-34-7-3	C	C-V
sow 9	h1	IV	32-53-34-4-3	155	C-III
pig 88	<d1	IV	35-48-35-8-4	E	C-I
	d1	IV	34-47-34-7-3	C	C-II
	d21	IV	35-59-35-8-4	E	C-I
	d3, d5, d17	IV	34-47-34-7-3	C	C-I
	d7	IV	34-47-33-7-3	36	C-I

[a] VNTR code of the repeat region of the 5 genes *clf*A, *clf*B, *sdr*C, *sdr*E and SIRU21

MLVA cluster A, the remaining MLVA types, the two pulsotypes, *spa* type and SCC*mec* type were unique to farm A (Table IV-4, Figure IV-9, Supplementary figure S1). Farms B, C and D shared one MLVA cluster (cluster B, dominant MLVA types: 33-57-37-7-3 and 33-57-37-6-3) and one pulsotype (Table IV-4, Figure IV-9). Moreover, MLVA cluster D (dominant MLVA type: 34-59-38-8-4) was similar on both farms C and D (Table IV-4). In general, PFGE and MLVA typing were more discriminatory than *spa* typing (one *spa* type versus few pulsotypes and few MLVA clusters). The pulsotype of one sow isolate, belonging to MLVA cluster A (sow 10, day 1) showed 87% similarity to the predominant pulsotype (data not shown). On farms C and D, the predominant pulsotype was detected in all MLVA clusters, present at the farm, and various other MLVA types. Isolates from MLVA clusters C and E

were categorized in the remaining four pulsotypes (less than 95% similar to the dominant type), detected on farm C, whereas MLVA clusters B and D in the other pulsotype (90% similarity with dominant pulsotype) on farm D (Supplementary table S2).

4.2 MLVA diversity on the low colonization farms (farms A and B)

The predominant MLVA cluster of both farms was found in isolates, originating from all units. On farm A, three out of four MRSA positive sows carried an MLVA type belonging to cluster A (Figure IV-4). The two positive sows of farm B (sows 4 and 12) were colonized with two non-related MLVA types (Figure IV-5). Their offspring were not necessarily colonized with the same or closely related MLVA type as the one the sow carried. For example, sow 12 (farm B) carried MLVA type 55 on the second sampling occasion. None of her offspring carried this or a closely related MLVA type (Figure IV-8A). After weaning, the piglets of farm A, originating from different litters, were mingled upon entry in the growing unit (one large pen), whereas on farm B, the litters remained together. Comparison of the MLVA types at the beginning and end of the growing period revealed the spread of some MLVA types throughout the cluster on farm A (Figure IV-8C). Novel MLVA types were also detected in the growing unit of which the majority belonged to the dominant MLVA cluster A (Figure IV-8C). The same observation (spread of MLVA types and novel MLVA types) was made in the finishing unit of both farms (Figure IV-8C). From birth to slaughter age, most animals carried various MLVA types, which in general belonged to the dominant cluster of the farm. Two out of four environmental isolates of farm A belonged to cluster A (Figure IV-4). On farm B, except for one, all environmental (wall) isolates belonged to cluster B (Figure IV-5).

4.3 MLVA diversity on the high colonization farms (farms C and D)

On the high colonization farms, piglets and their mother sows often carried MLVA types belonging to the same cluster. For example, sow 9 (farm D) carried MLVA types from cluster B. All four piglets carried cluster B MLVA types at least once in the nursing unit. Each piglet also carried MLVA types belonging to cluster E or unrelated types (Figure IV-8B). Piglets of the same litter did not always carry the same MLVA type or MLVA types of the same cluster at different sampling events. As seen on farms A and B, most animals carried several (closely related and unrelated) MLVA types throughout their life.

After a short stay in the growing unit, the pigs of farm C were transported to a second site where they resided until slaughter age. When observing the four dominant MLVA clusters of farm C, a shift in these clusters was observed when the pigs were transported to the second site: on site 1 clusters C and E were more predominantly present than clusters B and D, whereas on site 2 clusters B and D were almost exclusively present in the pigs (Figure IV-6B). On farm D, the two dominant MLVA clusters were equally found throughout the farm (Figure IV-7).

In most cases, the environmental isolates of farm C belonged to clusters B and C or these isolates belonged to singletons (MLVA types with only one isolate) (Figure IV-6A). More than half of the environmental isolates of farm D were situated in cluster B. Three MLVA types (6, 14 and 22) were similar to the sows and environment of this farm (Figure IV-7).

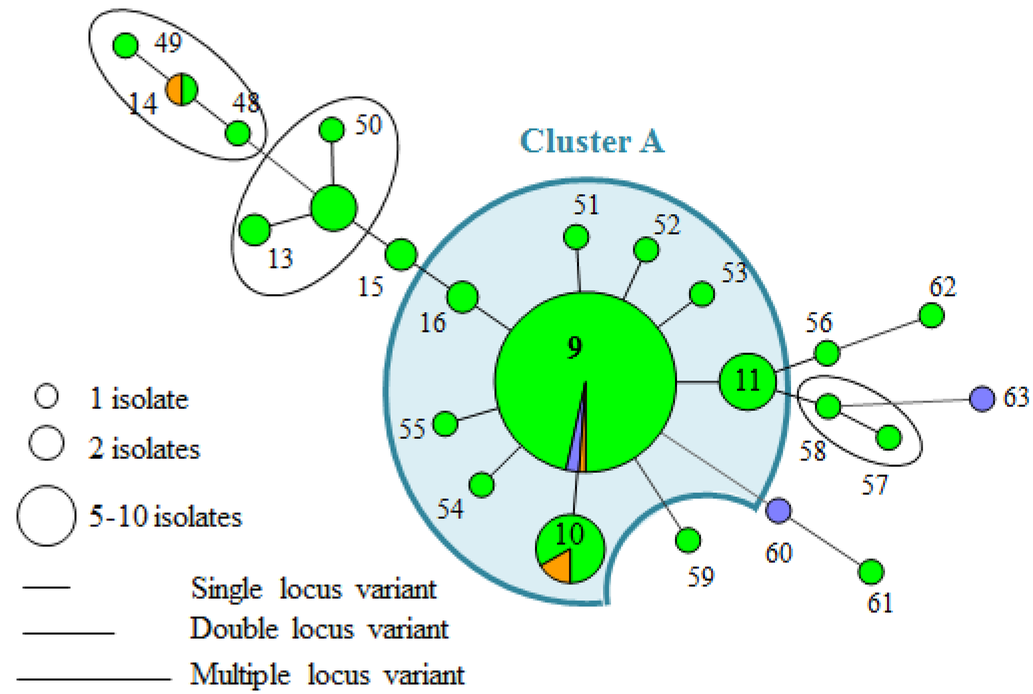


Figure IV-4 Minimum Spanning Tree of the farm A MLVA types, indicated by numbers, according to origin (green: piglet, orange: sow and blue: wall). The dominant cluster A of the farm is indicated in a coloured sphere, whereas the non-dominant clusters are indicated in black spheres.

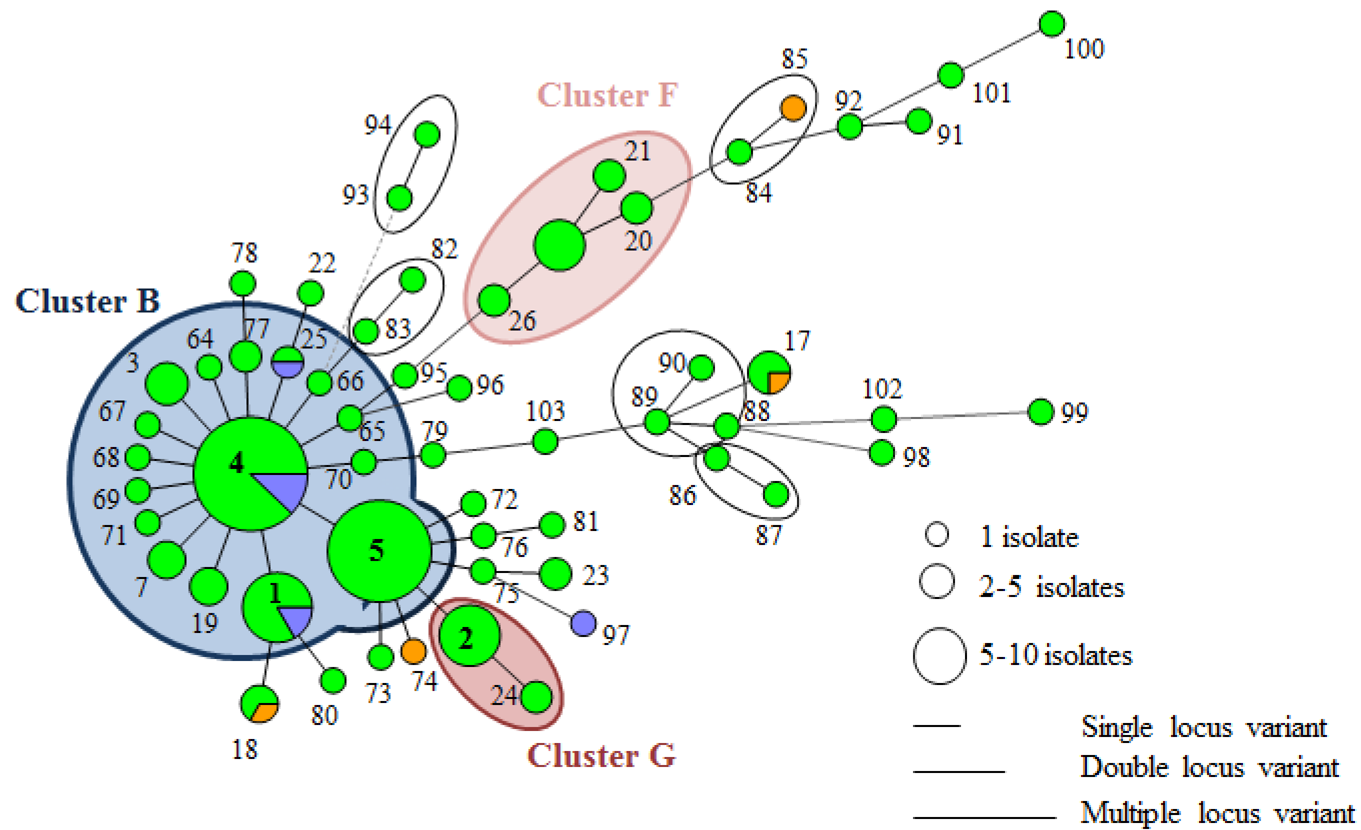


Figure IV-5 Minimum Spanning Tree of the farm B MLVA types, indicated by numbers, according to origin (green: piglet, orange: sow and blue: wall). The dominant clusters B, G and H of the farm is indicated in coloured spheres, whereas the non-dominant clusters are indicated in black spheres.

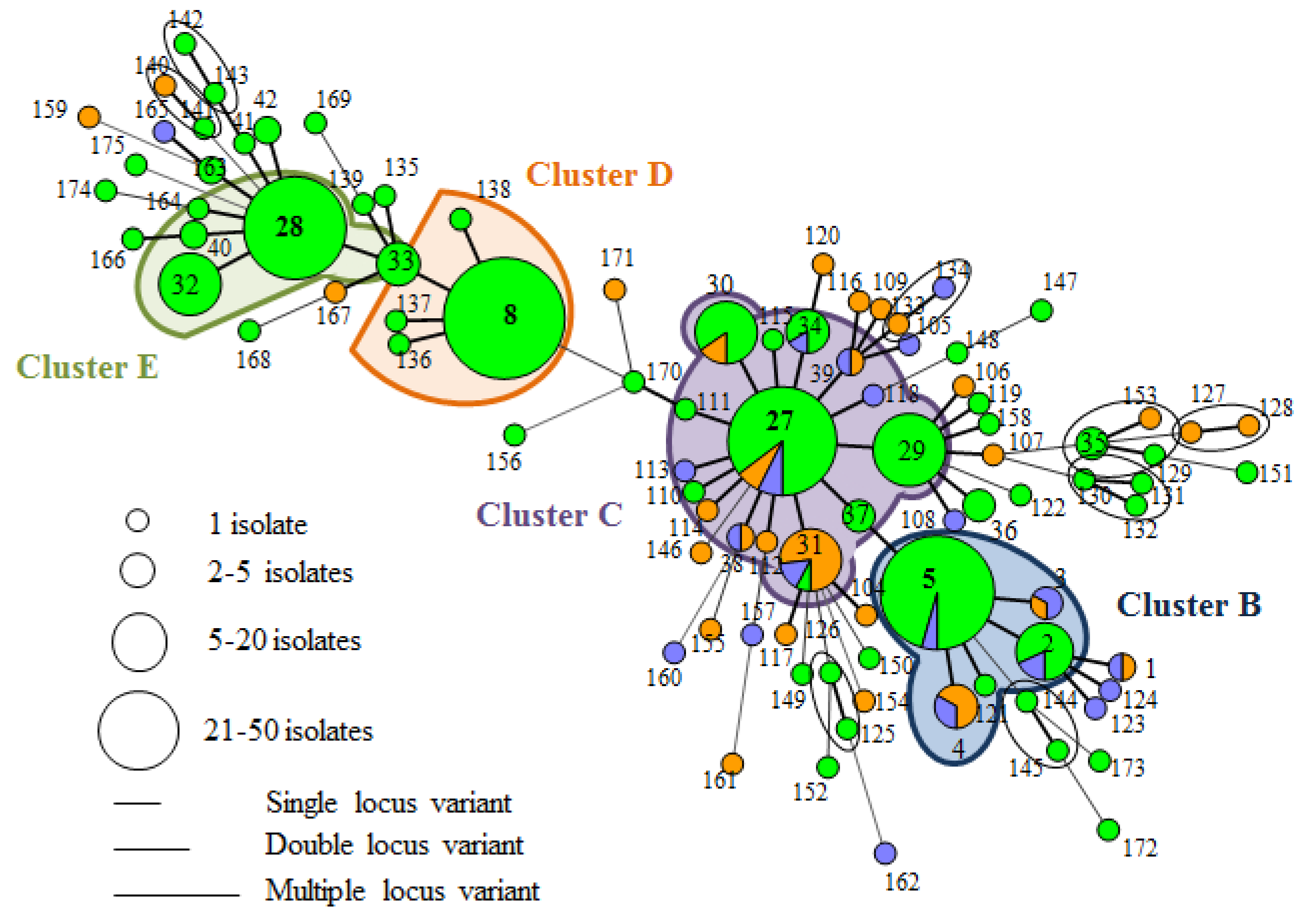


Figure IV-6A Minimum Spanning Tree of the farm C MLVA types, indicated by numbers, according to origin (green: piglet, orange: sow and blue: wall). The dominant clusters B, C, D and E of the farm is indicated in coloured spheres, whereas the non-dominant clusters are indicated in black spheres.

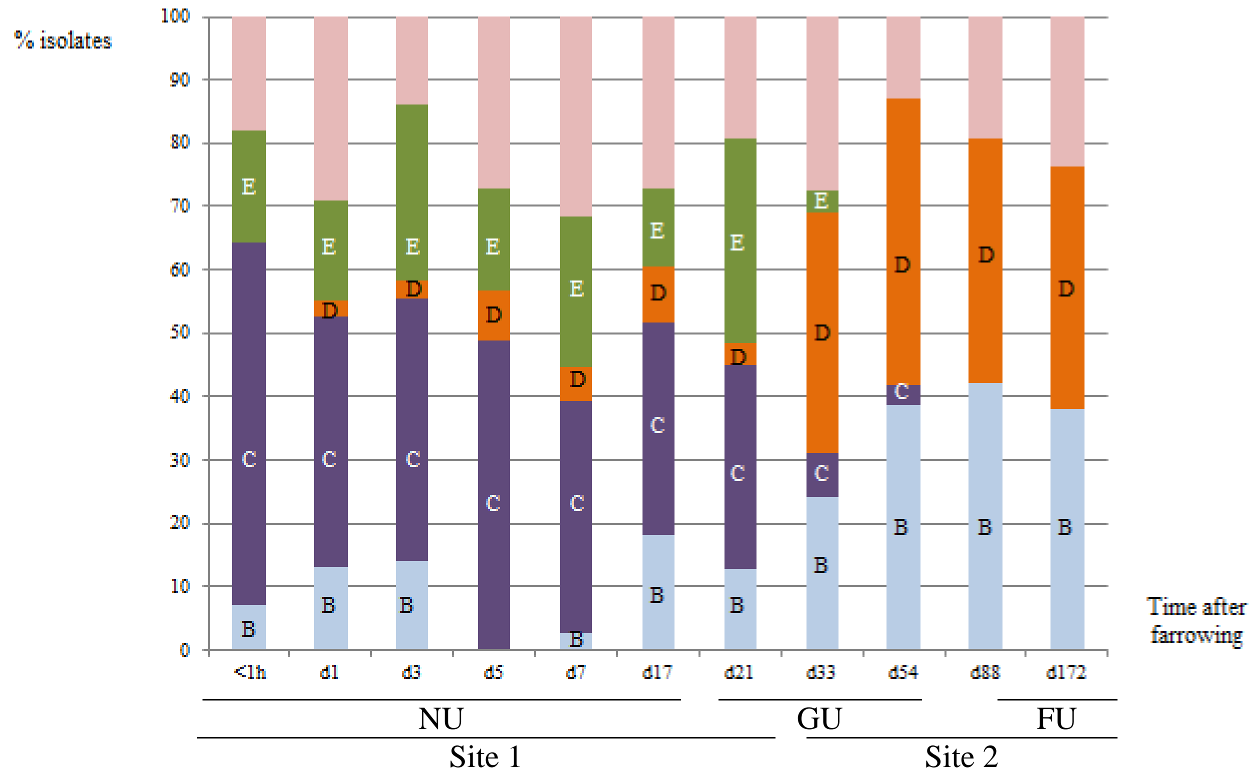


Figure IV-6B Distribution of the MLVA clusters at each time point after farrowing. The cluster designation is shown in the bar. Then non-dominant clusters and non-clustered MLVA types are given in the pink bar without indication. The piglets of farm C were transported to the second site after a short stay in the growing unit (h: hours, d: days, NU: nursing unit, GU: growing unit, FU: finishing unit)

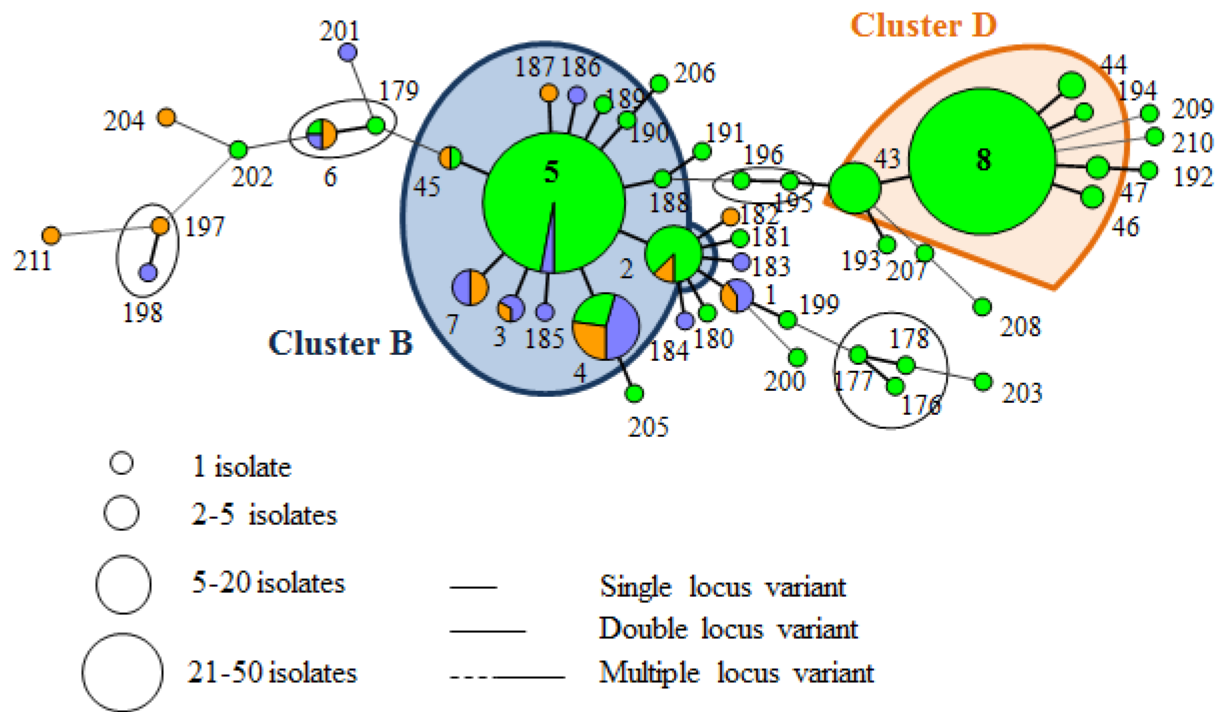
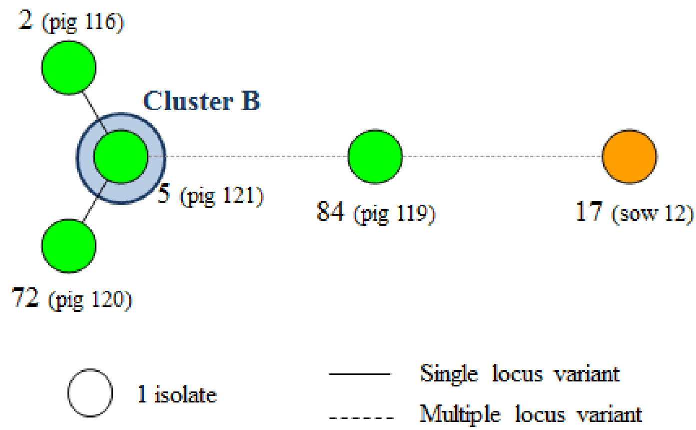
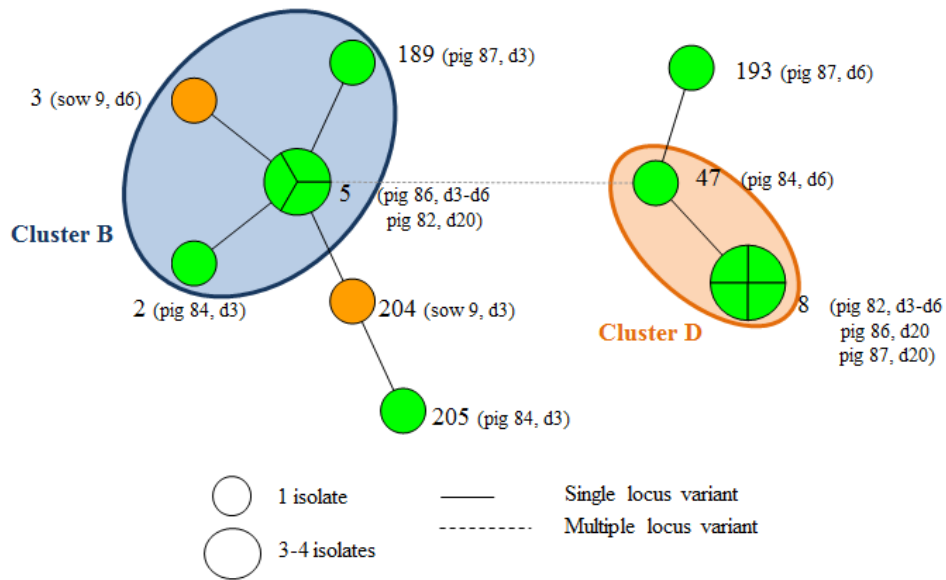


Figure IV-7 Minimum Spanning Tree of the farm D MLVA types, indicated in numbers, according to origin (green: piglet, orange: sow and blue: wall). The dominant clusters B and D of the farm are indicated in coloured spheres, whereas the non-dominant clusters are indicated in black spheres.

A



B



C

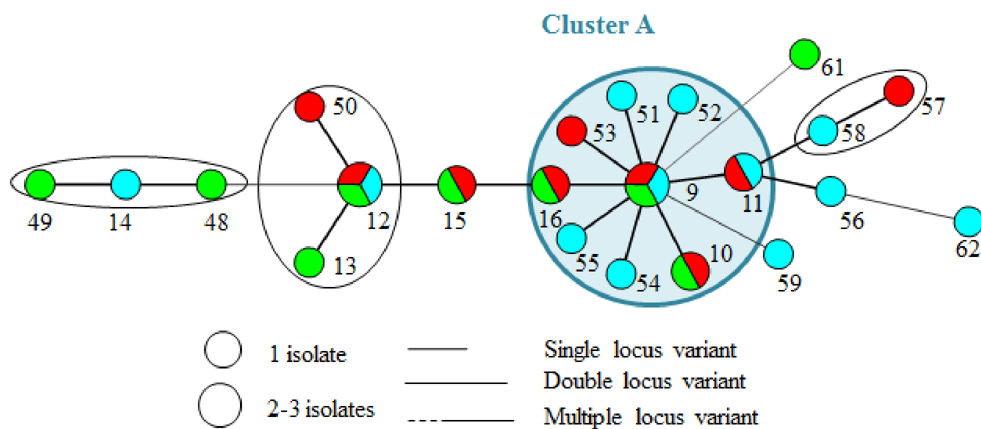
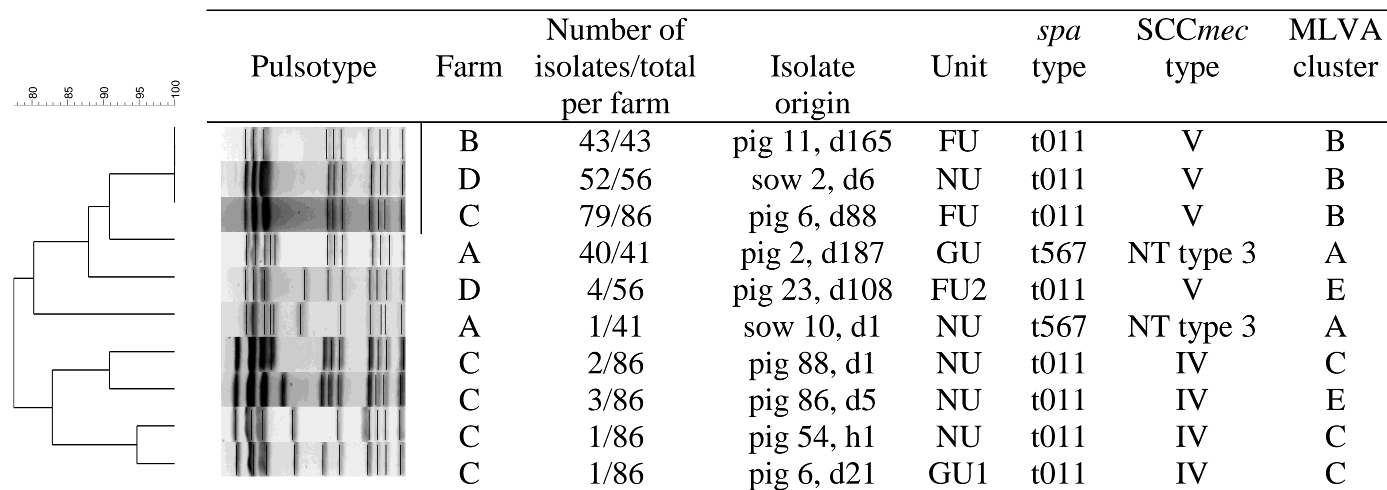


Figure IV-8 Overview of some observations. A) Piglets carrying non-related MLVA types to their mother sow (sow 12 and her offspring sampled one day after farrowing on farm B); B) Piglets carrying related MLVA types to their mother sow (sow 9 and her offspring sampled 3, 6 and 20 days after farrowing on farm D). In addition, piglets of the same litter carried various related and unrelated MLVA types; C) Overview of the MLVA types found in the three units of farm A (green: nursing unit, red: growing unit and blue: finishing unit).



Pulsotype	Farm	Number of isolates/total per farm	Isolate origin	Unit	*spa* type	SCC*mec* type	MLVA cluster
	B	43/43	pig 11, d165	FU	t011	V	B
	D	52/56	sow 2, d6	NU	t011	V	B
	C	79/86	pig 6, d88	FU	t011	V	B
	A	40/41	pig 2, d187	GU	t567	NT type 3	A
	D	4/56	pig 23, d108	FU2	t011	V	E
	A	1/41	sow 10, d1	NU	t567	NT type 3	A
	C	2/86	pig 88, d1	NU	t011	IV	C
	C	3/86	pig 86, d5	NU	t011	IV	E
	C	1/86	pig 54, h1	NU	t011	IV	C
	C	1/86	pig 6, d21	GU1	t011	IV	C

Figure IV-9 Dendrogram containing the pulsotypes obtained on the four farms. Per pulsotype, one isolate is shown as an example. Consecutive the dendrogram, pulsotype pattern, farm and number of isolates belonging to the pulsotype on the total number of typed isolates per farm are shown. For each example, the isolate origin, unit (NU: nursing unit, GU: growing unit, GU1: growing unit 1, FU: finishing unit, FU2: finishing unit 2), *spa* type, SCC*mec* type (NT type 3: *mec*A complex NT/*ccr* complec C) and MLVA cluster are given.

5 Discussion

At present, many molecular typing methods are available for the characterization of MRSA ST398. Seen the clonal nature of this MRSA type when using MLST, PFGE and *spa* typing, the use of other more discriminatory methods should be considered (Weese et al., 2010a). A more recently optimized method for MRSA ST398 typing is MLVA (Rasschaert et al., 2009). To our knowledge, this is the first study where MLVA was used on a large collection of MRSA ST398 isolates from pigs.

Upon comparison of the typing methods, used during the present study, an important difference was observed between the results. After *spa* typing, SCC*mec* typing and PFGE approximately one or a few genotype(s) per farm was found. In contrast, a large and unexpected variety in MLVA types was observed on each farm (224 MLVA types in total) after initial analysis of the results. During this analysis, one difference in a repeat region was considered as a new MLVA type as recommended by Applied Maths (personal communication). A first explanation for this variety could be the MLVA method itself. Small variations within the assignment of the repeat numbers could have occurred, resulting in different MLVA types. However, during each run, the same positive control was used and the same results were observed each time for this strain. So, the method had a good inter- and intra repeatability (data not shown). Another possible explanation for the observed variety is situated in the analyzed loci. It is possible that the repeat regions of the five analyzed loci are less stable and evolve faster than the loci studied during *spa* typing and PFGE. It has been reported that repeat regions of, for example surface proteins, evolve really fast, resulting in various related isolates, which was also seen here (Bhaya et al., 2011; Wang et al., 2011; Westra et al., 2012). However, when these loci evolve too fast, this could result in a "too highly" discriminatory method, which is a possibility that should be further elucidated. Other research groups have described other MLVA typing schemes and more importantly, other interpretation methods (for example: using a cut-off value or various settings in the computer programs upon clustering of the results). Quite often regular gel electrophoresis was used instead of capillary electrophoresis, which makes comparison with these reports difficult (Sabat et al., 2003; Schouls et al., 2009; Holmes et al., 2010). Here, the predominant MLVA type(s) of a farm was(were) clustered with their single locus variants, which resulted in less MLVA variety. Clustering of the MLVA types allowed us to interpret the results better. In

addition, when comparing the typing results, MLVA appeared highly discriminatory for MRSA ST398 isolates.

Nevertheless, caution is needed upon combination of the typing results. *Spa* typing, PFGE and MLVA are methods that are used to detect relationships between the isolates. For example, when generating the MST (MLVA results), the most dominant type is considered as the basal or ancestral type. Subsequently, the remaining MLVA types are positioned according to their differences in repeat regions. When using SCC*mec* typing, the horizontal gene transfer of these cassettes is studied. On farm C, two SCC*mec* cassettes were found, which indicates that two separate transfers have occurred. Both cassettes were found in different MLVA clusters (type IV in clusters C/E and type V in clusters B/D). This would mean that the proposed relationships between the MLVA types are incorrect and should be adapted. However, seen the limited number of isolates that underwent SCC*mec* typing, more typing is needed to investigate this.

The main objective of the present work was to gain insights into the genetic diversity of LA-MRSA isolates, originating from a farm. Remarkably, one genotype (same *spa*, pulsotype and MLVA cluster) was common on farms B, C and D. Direct carry-over of LA-MRSA between these farms could be excluded (geographical distance, different veterinarian, no direct exchange of animals) (Verhegghe et al., 2013a). The *spa* type on these farms was t011, which is a more widely distributed MRSA ST398 strain in pig farms than others. It is also possible that farm specific or region specific genotypes are present, since the genotype, found on farm A was unique to this farm. Since only four farms were sampled, additional samplings are needed to elucidate these possibilities.

On each farm, a few dominant genotypes were isolated from the animals and their environment. It appears that these dominant types persist better within the farrow-to-finish farm. A good indication of this persistence was seen on farm C. Four clusters were observed on this farm and all clusters were present on both sites, but the isolation percentage of the clusters was not equal on both sites: clusters B and D were isolated from less than 10% of the isolates on site 1, whereas from 30 to 40% of the isolates on site 2. Further research is needed to determine the exact mechanism for the observed persistence.

Another interesting observation after MLVA typing is that the sows did not carry the most prevalent MLVA types, as observed in their offspring. It might be possible that these dominant MLVA types are age-specific. However, compared to the number of piglet isolates,

fewer sow isolates were obtained, so, additional sow samplings and typing are needed to confirm the presence of age-specific MLVA types.
Besides the dominant MLVA types, all pigs carried another MLVA type at least once throughout their lifespan. These additional types were either related or non-related to the dominant type. Plausible explanations for these results are that some MLVA types are transition types to another type; that the animals are intermittent carriers of certain MLVA types or carriers of other MLVA types besides the dominant type. Because only one isolate per animal and per sampling event was analyzed, these hypotheses cannot be confirmed from the present data. In addition, the isolation method used in the study may have been insufficient to detect all MRSA present on the animals (Verhegghe et al., 2013a).

A second objective was to determine potential MRSA sources for the animals present at the farm. In general, a few dominant and widespread genotypes were detected in the animals and environment of the farm. Since a farrow-to-finish farm can be considered as a closed system (few animals are imported), this would mean that the farm as it whole can act as a source for newborn piglets. Using MLVA typing helped to clarify three possible LA-MRSA sources for pigs. First, in general, none of the piglets carried an identical MLVA type to its mother sow at farrowing. Nevertheless, in most cases, the MLVA types of mother sows and their offspring were closely related and belonged to the same cluster. This could be explained by the fast occurrence of mutations in the repeat regions of the five genes when the mother sow strains colonize their offspring. Moreover, Crombé and co-workers (2012c) reported on the presence of maternal antibodies in piglets, which puts forward the possibility of piglets being immune to the mother sow MLVA type, but not to the closely related type. Second, the dominant MLVA clusters were also found in the environment of the piglets, which could be considered as an additional source for the animals. Moreover, transmission between animals and their environment and *vice versa* was already suggested and demonstrated by other research groups (Gibbons et al., 2013; Pletinckx et al., 2013a). Third, when mingling pigs upon relocation in other units, spread of some MLVA types throughout the group occurred, which confirms that the pig(let)s themselves act as a MRSA source. This was already reported in various colonization experiments (Broens et al., 2012b; Crombé et al., 2012b).

6 Conclusion

During the present study, isolates of animal and environmental origin were studied, using MLVA typing, PFGE, *spa* typing and SCC*mec* typing. The latter three methods demonstrated the clonal properties of the isolates, but more variation was observed when using MLVA typing. One genotype was similar on three farms, which could indicate that one LA-MRSA clone is more widespread than others. Within a farm, a few dominant genotypes were present (one pulsotype, one *spa* type, one SCC*mec* type and a few MLVA clusters), which were widespread. Potential MRSA sources for piglets were the mother sows, the environment and other piglets. In conclusion, a farrow-to-finish farm can be considered as a closed system in which a dominant MRSA clone persists.

7 Acknowledgements

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Supplementary data

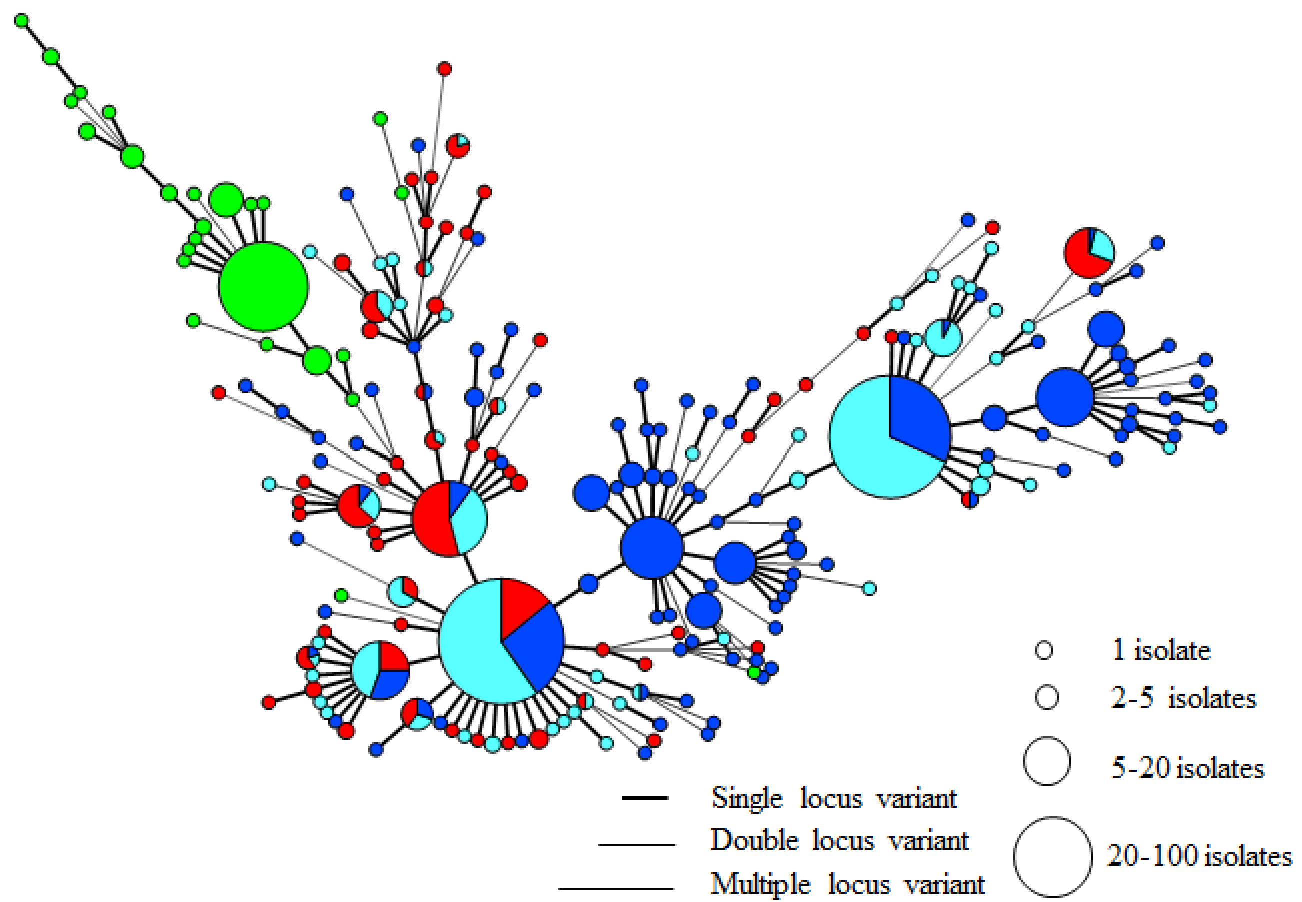

Figure S1: Minimum Spanning Tree of the isolates, originating from all farms (farm A: green, farm B: red, farm C: light blue and farm D: dark blue).

Table S1 Overview of the obtained MLVA results of the isolates originating from the four farms (A-D). The MLVA types occurring on three or two farms are shown first. Afterwards, the MLVA types are ordered from farm A to D. For each MLVA type, present at the farm, the number of isolates per origin is shown. The last column indicates the isolate percentage per farm, present in the MLVA type. The pigs of farm C were transported from site 1 (C1) to site 2 (C2). The sows of farm C were only present on site 1.

MLVA numeric code[a]	MLVA Type	Number of isolates per isolate origin														Isolate percentage (%)			
		Sow				Pig					Wall								
		A	B	C	D	A	B	C_1	C_2	D	A	B	C_1	C_2	D	A	B	C	D
33-57-36-6-3	1		0	1	2		10	0	0	0		2	0	1	3		7.7	0.6	1.5
33-57-36-7-3	2		0	0	2		9	2	7	14		0	0	2	0		5.8	3.7	4.9
33-57-37-5-3	3		0	2	1		4	0	0	0		0	1	1	2		2.6	1.1	0.9
33-57-37-6-3	4		0	4	6		29	0	0	6		4	0	2	13		21.3	1.6	7.6
33-57-37-7-3	5		0	0	0		24	15	28	99		0	0	2	0		15.5	12.7	30.4
32-55-35-5-3	6		0		2		8			1		0			1		5.2		1.2
33-57-37-4-3	7		0		3		3			0		0			3		1.9		1.8
34-59-38-8-4	8			0	0			11	40	110			0	0	0			14.4	33.7
32-46-38-38-2	9	1				83					2					63.7			
32-46-39-38-2	10	2				10					0					8.9			
32-46-38-38-3	11	0				8					0					5.9			
32-45-37-37-2	12	0				5					0					3.7			
31-45-37-37-2	13	0				2					0					1.5			
32-44-36-36-2	14	1				1					0					1.5			
32-46-37-37-2	15	0				2					0					1.5			
32-46-38-37-2	16	0				2					0					1.5			
31-53-34-1-2	17		0				3					1					2.6		
33-57-36-4-3	18		1				2					0					1.9		
33-57-37-3-3	19		0				3					0					1.9		
32-55-35-3-3	20		0				2					0					1.3		
32-55-35-4-3	21		0				2					0					1.3		
33-47-37-6-3	22		0				2					0					1.3		
33-51-36-7-3	23		0				2					0					1.3		
33-52-36-7-3	24		0				2					0					1.3		
33-57-35-6-3	25		0				1					1					1.3		
32-53-35-5-3	26		0				2					0					1.3		

MLVA numeric code[a]	MLVA Type	Number of isolates per isolate origin																	
		Sow				Pig					Wall					Isolate percentage (%)			
		A	B	C	D	A	B	C1	C2	D	A	B	C1	C2	D	A	B	C	D
34-57-34-7-3	27			3				34	1				3	0				11.5	
35-59-35-8-4	28			0				30	1				0	0				8.7	
34-57-33-7-3	29			0				17	1				0	0				5.1	
34-47-34-7-3	30			2				11	0				0	0				3.7	
34-57-34-6-3	31			10				1	0				2	0				3.7	
35-48-35-8-4	32			0				13	0				0	0				3.7	
35-59-38-8-4	33			0				3	3				0	0				1.6	
34-57-34-7-1	34			0				5	0				0	0				1.4	
33-55-33-6-3	35			0				3	0				0	0				0.8	
34-47-33-7-3	36			0				3	0				0	0				0.8	
34-57-37-7-3	37			0				3	0				0	0				0.8	
34-57-34-4-3	38			1				0	0				0	1				0.6	
34-57-34-5-3	39			1				0	0				1	0				0.6	
35-58-35-8-4	40			0				2	0				0	0				0.6	
35-59-35-8-2	41			0				2	0				0	0				0.6	
35-59-35-8-3	42			0				2	0				0	0				0.6	
34-58-38-8-4	43				0					13					0				4.0
34-59-38-8-3	44				0					3					0				0.9
33-55-37-7-3	45				1					1					0				0.7
34-57-38-8-4	46				0					2					0				0.7
34-59-38-7-4	47				0					2					0				0.7
Others[b]	48-212	0	2	21	5	14	35	40		25	2	1	11		6	11.8	24.5	20.3	11.0

[a]VNTR code of the repeat region of the 5 genes c*lf*A, c*lf*B, *sdr*C, *sdr*E and SIRU21)

[b]MLVA types containing only one isolate being on farm A (MLVA types 48-63): 32-45-36-36-2; 30-44-36-36-2; 25-46-38-38-2; 30-46-38-38-2; 31-46-38-38-2; 32-49-38-38-2; 32-46-38-38-1; 32-46-39-38-3; 33-46-38-37-3; 33-46-38-38-3; 38-46-38-38-4; 32-43-33-33-2; 32-43-35-34-2; 25-40-39-38-3 and 33-44-34-34-3; on farm B (MLVA types 64-103): 33-57-37-38-3; 33-53-37-6-3: 33-52-37-6-3; 33-50-37-6-3; 33-48-37-6-3; 33-49-37-6-3; 33-55-37-6-3; 33-46-37-6-3; 33-46-37-7-3; 33-47-37-7-3; 33-41-37-7-3; 33-53-37-7-3; 33-51-37-7-3; 32-57-35-6-3; 33-47-36-6-3; 33-55-36-6-3; 32-57-36-6-3; 33-53-36-7-3; 33-52-36-2-3; 33-52-36-6-3; 31-54-

35-3-3; 31-54-35-4-3; 32-55-35-1-2; 31-55-35-1-2; 32-51-34-1-2; 32-55-34-1-2; 32-55-34-2-2; 31-53-34-2-3; 31-53-34-3-3; 34-52-38-8-4; 34-48-38-8-4; 33-53-37-5-3; 33-53-33-8-3; 33-51-30-9-3; 26-51-32-2-2; 27-50-32-0-2; 29-52-33-3-2; 31-53-33-3-2; 28-51-33-0-2 and 33-55-36-1-2; on farm C (MLVA types 104-176): 34-57-37-6-3; 34-57-37-5-3; 34-57-33-4-3; 34-57-33-6-3; 34-57-33-3-3; 34-57-33-5-3; 34-57-36-7-3; 34-57-34-8-3; 34-57-34-2-3; 34-57-34-3-3; 34-53-34-7-3; 34-58-34-7-3; 34-47-34-5-3; 34-51-34-6-3; 34-51-34-7-3; 34-57-33-7-1; 34-48-34-7-1; 33-58-37-7-3; 33-57-33-7-3; 33-57-36-4-3; 33-57-36-5-3; 32-55-35-6-3; 32-57-35-6-3; 33-55-35-1-3; 33-55-35-0-3; 33-46-33-6-3; 27-51-33-6-3; 27-49-33-6-3; 27-50-33-6-3; 34-57-34-5-2; 35-57-34-5-2; 35-58-38-8-4; 34-58-38-8-4; 34-52-38-8-4; 34-56-38-8-4; 26-59-38-8-4; 31-53-33-8-4; 31-53-35-8-4; 36-62-39-8-4; 35-39-8-4; 33-57-37-8-4; 33-57-36-8-4; 34-57-39-11-3; 29-52-32-7-3; 28-51-32-7-3; 28-49-34-6-3; 29-50-34-6-3; 24-46-30-6-3; 31-53-35-6-3; 33-55-33-2-3; 24-57-32-6-1; 32-53-34-4-3; 32-54-34-9-4; 34-58-34-2-2; 34-57-33-8-2; 36-59-34-1-2; 30-57-31-4-2; 29-39-30-2-2; 32-55-32-3-2; 35-59-39-8-4; 35-59-35-9-4; 35-47-35-8-2; 35-58-35-7-4; 35-50-38-8-4; 36-50-36-8-4; 26-49-30-8-4; 34-57-34-8-4; 33-53-34-8-4; 33-57-38-38-4; 41-66-37-8-4; 40-65-35-9-4 and 35-59-40-40-6; on farm D (MLVA types 177-212): 32-54-34-6-3; 32-55-34-6-3; 32-52-34-6-3; 32-55-35-7-3; 33-54-36-7-3; 33-58-36-7-3; 33-57-36-4-3; 33-57-36-3-3; 28-57-36-7-3; 33-51-37-7-3; 33-50-37-7-3; 33-57-37-1-3; 33-57-37-8-3; 32-57-37-7-3; 33-57-37-7-4; 33-57-37-8-4; 34-59-37-7-4; 34-58-38-7-4; 34-59-38-8-2; 34-58-38-8-3; 33-58-38-8-3; 29-52-33-1-2; 28-52-33-1-2; 33-55-36-6-3; 34-58-36-6-3; 32-55-35-1-2; 28-52-35-5-3; 29-52-34-7-3; 29-58-33-5-3; 33-57-35-6-3; 35-57-37-7-4; 33-58-35-8-4; 32-53-35-8-4; 31-55-34-8-4; 40-65-38-8-5 and 31-53-34-1-2.

Table S2 Results of the four performed molecular typing methods on a selection of isolates, originating from the four farms (A-D). For each isolate, the farm of origin, isolate origin, MLVA numeric code, MLVA cluster or MLVA type, pulsotype, *spa* type and SCCmec type is shown (d: days, h: hour).

Farm	Isolate origin	MLVA numeric code[a]	MLVA cluster/type[b]	Pulsotype	*Spa* type	SCC*mec* type
A	pig 2, d187	32-46-38-38-2	A	A-I	t567	NT type 3
A	pig 51, d3	32-46-39-38-2	A	A-I	t567	NT type 3
A	pig 51, d5	32-46-39-38-2	A	A-I	t567	NT type 3
A	pig 2, d7	32-45-36-36-2	48	A-I	t567	NT type 3
A	sow 6, d7	32-44-36-36-2	14	A-I	t567	NT type 3
A	pig 51, d7	30-44-36-36-2	49	A-I	t567	NT type 3
A	pig 2, d52	32-46-38-38-2	A	A-I	t567	NT type 3
A	pig 51, d52	32-46-38-38-2	A	A-I	t567	NT type 3
A	pig 2, d75	32-46-38-38-2	A	A-I	t567	NT type 3
A	pig 51, d75	32-46-38-38-2	A	A-I	t567	NT type 3
A	sow 10, d1	32-46-39-38-2	A	A-II	t567	NT type 3
B	sow 4, h1	31-54-35-4-3	85	BI	t011	V
B	pig 40, d1	33-57-36-6-3	B	B-I	t011	V
B	pig 83, d1	33-57-37-7-3	B	B-I	t011	V
B	pig 40, d3	28-51-33-0-2	102	B-I	t011	V
B	pig 83, d3	32-55-35-5-3	F	B-I	t011	V
B	sow 4, d5	33-57-36-4-3	F	B-I	t011	V
B	pig 40, d58	33-48-37-6-3	B	B-I	t011	V
B	pig 83, d58	33-57-37-3-3	B	B-I	t011	V
B	pig 18, d67	32-57-36-6-3	80	B-I	t011	V
B	pig 40, d67	33-57-37-4-3	B	B-I	t011	V
B	pig 83, d165	32-55-34-2-2	90	B-I	t011	V
C	sow 1, d3	33-57-37-6-3	B	C-I	t011	V
C	sow 1, d17	33-57-36-6-3	1	C-I	t011	V
C	pig 6, d1	33-57-37-7-3	B	C-I	t011	V
C	pig 6, d3	33-57-36-7-3	B	C-I	t011	V
C	pig 6, d5	34-59-38-8-4	D	C-I	t011	V
C	pig 6, d7	31-53-35-6-3	152	C-I	t011	V
C	pig 6, d17	34-59-38-8-4	C	C-I	t011	V

Farm	Isolate origin	MLVA numeric code[a]	MLVA cluster/type[b]	Pulsotype	*Spa* type	SCC*mec* type
C	pig 6, d33	34-59-38-8-4	C	C-I	t011	V
C	pig 6, d54	33-57-37-7-3	B	C-I	t011	V
C	pig 6, d88	33-57-37-7-3	B	C-I	t011	V
C	pig 88, d33	33-57-37-7-3	B	C-I	t011	V
C	pig 88, d54	34-59-38-8-4	D	C-I	t011	V
C	pig 88, d88	34-59-38-8-4	D	C-I	t011	V
C	pig 88, d172	26-59-38-8-4	139	C-I	t011	V
C	sow 1, d1	33-55-35-1-3	127	C-I	t011	IV
C	sow 1, d5	33-53-34-8-4	171	C-I	t011	IV
C	pig 6, d21	34-57-34-7-3	C	C-V	t011	IV
C	sow 9, h1	32-53-34-4-3	155	C-III	t011	IV
C	pig 88, h1	35-48-35-8-4	E	C-I	t011	IV
C	pig 88, d1	34-47-34-7-3	D	C-II	t011	IV
C	pig 88, d3	34-47-34-7-3	D	C-I	t011	IV
C	pig 88, d5	34-47-34-7-3	D	C-I	t011	IV
C	pig 88, d7	34-47-33-7-3	D	C-I	t011	IV
C	pig 88, d17	34-47-34-7-3	D	C-I	t011	IV
C	pig 88, d21	35-59-35-8-4	163	C-I	t011	IV
D	pig 20, d3	33-57-37-6-3	B	D-I	t011	V
D	sow 1, d6	32-55-35-5-3	6	D-I	t011	V
D	sow 2, d6	33-57-37-6-3	B	D-I	t011	V
D	pig 20, d6	33-57-37-7-3	B	D-I	t011	V
D	sow 3, d6	33-57-37-6-3	B	D-I	t011	V
D	pig 23, d6	33-57-36-7-3	B	D-I	t011	V
D	pig 100, d6	33-55-37-7-3	B	D-I	t011	V
D	sow 1, d20	33-57-36-6-3	1	D-I	t011	V
D	sow 3, d20	33-57-36-4-3	182	D-I	t011	V
D	pig 23, d20	34-59-38-8-4	D	D-I	t011	V
D	pig 4, d27	33-57-37-7-3	B	D-I	t011	V
D	pig 20, d27	33-58-35-8-4	207	D-I	t011	V
D	pig 23, d27	33-55-36-6-3	199	D-I	t011	V

Farm	Isolate origin	MLVA numeric code[a]	MLVA cluster/type[b]	Pulsotype	*Spa* type	SCC*mec* type
D	pig 4, d35	33-57-37-7-3	B	D-I	t011	V
D	pig 20, d35	34-59-38-8-4	D	D-I	t011	V
D	pig 23, d35	34-59-38-8-4	D	D-I	t011	V
D	pig 20, d62	33-57-37-7-3	B	D-I	t011	V
D	pig 23, d77	33-57-37-7-3	B	D-I	t011	V
D	pig 20, d159	33-57-37-7-3	B	D-I	t011	V
D	pig 23, d159	33-57-37-7-3	B	D-I	t011	V
D	pig 23, d48	34-59-38-8-4	D	D-II	t011	V
D	pig 23, d62	34-59-38-8-4	D	D-II	t011	V
D	pig 20, d108	32-55-35-7-3	179	D-II	t011	V
D	pig 23, d108	33-57-37-7-3	B	D-II	t011	V

[a] VNTR code of the repeat region of the 5 genes *clf*A, *clf*B, *sdr*C, *sdr*E and SIRU21

[b] In case of a clustered MLVA type, the cluster letter is given. In case of a non-clustered MLVA type, the unique MLVA number is given.

Chapter V. A. Best sampling location on a pig carcass for livestock-associated MRSA

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1 Abstract

In the present study, the optimal sampling location for LA-MRSA on pig carcasses was determined. In one slaughterhouse, 40 cooled carcasses from one LA-MRSA-positive herd were sampled on six parts of the carcass (ham, abdomen, back, forelimb, sternum and intestinal cavity). From each location, 100 cm² was swabbed using a pre-moistened sponge. Each sponge was diluted ten times in salt-enriched broth and this dilution was inoculated on Chrom-ID™ MRSA. Subsequently, ten-fold dilution series in the same broth were made and incubated overnight. One hundred µl of each dilution was spread plated onto Chrom-ID™ MRSA. Suspect colonies were confirmed by a MRSA and CC398 specific PCR. Carcass isolates were characterized by Pulsed Field Gel Electrophoresis (PFGE) using *Bst*ZI restriction and pulsotypes were compared with the strains obtained from the live animals of the herd.

No MRSA was detected after direct inoculation on a selective agar medium. After enrichment, MRSA ST398 was isolated from 19 out of 40 (48%) carcasses and in 16 cases from the forelimb. All obtained isolates belonged to CC398. Three pulsotypes were detected and the predominant pulsotype was also the herd pulsotype.

In conclusion, on approximately half of the carcasses MRSA ST398 was isolated, but low numbers of this bacterium were expected to be present. Moreover, the forelimb, a less-frequently consumed part of the carcass, appeared to be the most contaminated part of the carcass. The recovery of the herd pulsotype on the carcasses indicated that cross-contamination from the animals on the carcass may occur. Recovery of non-herd pulsotypes (encountered in 18% of the contaminated carcasses) indicates that MRSA are widespread along the production line. Still, additional sampling events on carcasses in other slaughterhouses are needed to confirm the results and to determine possible transmission routes.

2 Introduction

Since the first description of livestock-associated MRSA (LA-MRSA) in The Netherlands, this MRSA type has been found worldwide in different livestock animals and humans working with those animals (Voss et al., 2005; Vanderhaeghen et al., 2010a). Livestock animals are mostly carriers of LA-MRSA and are therefore considered as a potential reservoir for the general human population. Besides direct contact with live animals, a possible exposure route for LA-MRSA is thought to be meat. At present, low numbers of MRSA have been found on meat, indicating a possible but low exposure risk to the human population (de Boer et al., 2009; Van Loo et al., 2007; Weese et al., 2010c).

A pig carcass might get contaminated with LA-MRSA at the slaughterhouse. For the detection of pathogens such as *Salmonella*, control samplings occur on a two-weekly basis at the slaughterhouse (European Commission, 2005). During these sampling events, six locations are sampled. This sampling plan is of great importance to detect a possible flow of the pathogens to the human population. To our knowledge, no guidelines are available for the detection of LA-MRSA on pig carcasses. In the present study, the best sampling location for MRSA on a pig carcass at the slaughterhouse was determined based on the *Salmonella* protocol. This will be helpful to determine the types of pork meat to screen for LA-MRSA prevalence. Moreover, Pulsed Field Gel Electrophoresis (PFGE) was performed to gain insight into the genetic variety of the obtained isolates.

3 Material and methods

3.1 Sampling methodology and processing

Sampling was performed in one slaughterhouse, located in the northern part of Belgium. Approximately two hours after slaughter, 40 carcasses of a MRSA-positive herd consisting of 120 animals were sampled in the cooling room (Verhegghe et al., 2013a). Nineteen right-carcass halves and 21 left-carcass halves were randomly chosen. The carcasses were sampled at six places as described by Ghafir et al. (2005) (Figure V-1). The outside of the carcass was sampled at the ham, the abdomen and the back. Sampling of the inner part consisted of the inner side of the forelimb, the sternum and the abdominal cavity. From each sampling location, 100 cm² was swabbed with an envirosponge (3M Dry Sponge; BP133ES; Led Techno; St. Paul, MN, US), premoistened with salt-enriched (6.5%; Sodium chloride;

1.06404; Merk, Darmstadt, DE) Mueller Hinton Broth (MHB CM0405; Oxoid, Basingstoke, UK). All samples were transported and processed immediately upon arrival at the laboratory (two to three hours after the sampling event). Salt-enriched MHB was added to the sponges to obtain a 9/1 ratio and sponges were mixed manually for 30 seconds. A ten-fold dilution series of this enrichment broth was made in salt enriched MHB up to dilution 10^{-3}. One hundred µl of the original enrichment broth was spread-plated onto a chromogenic selective medium for MRSA (Chrom-ID™ MRSA; BioMerieux, Marcy l'Etoile, FR) after which the plates were incubated overnight (18-20h, 37°C). After overnight incubation (37°C for 18-20h), the dilution series were spread-plated and incubated onto Chrom-ID™ MRSA as described above. One suspect colony per plate, obtained after incubation, was purified onto a Chrom-ID™ MRSA plate. Pure isolates were stored at -20°C in brain-heart infusion broth (BHI; CM0225; Oxoid, Basingstoke, UK) supplemented with glycerol (15% wt/vol; Fisher Scientific, Leicestershire, UK).

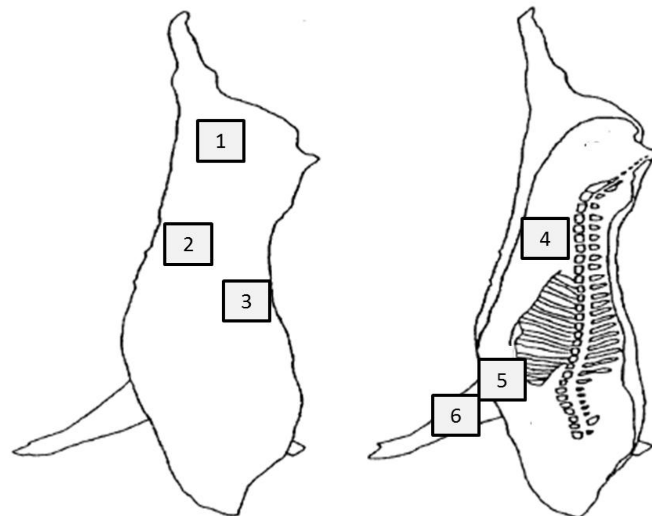

Figure V-1 The different locations on the carcass (1: the ham, 2: the abdomen, 3: the back, 4: the intestine cavity, 5: the sternum, 6: the inner part of the forelimb).

3.2 MRSA confirmation and Pulsed Field Gel Electrophoresis (PFGE)

From each isolate, DNA was extracted according to Strandén et al. (2003) and then stored at -20°C until further use. An MRSA-specific multiplex PCR confirmed the presence of MRSA (Maes et al., 2002). A carcass was considered to be MRSA-positive if MRSA was isolated from at least one location. On all obtained isolates, a CC398-specific PCR and PFGE using *Bst*ZI (Promega, Madison, WI, US) as a restriction enzyme were performed as described by Stegger et al. (2011) and Rasschaert et al. (2009), respectively. The obtained restriction profiles were analyzed with Bionumerics version 6.5 (Applied Maths, St.-Martens-Latem,

BE) using the unweighted pair group method using averages (UPGMA) with the Dice coefficient (tolerance 1%, tolerance change 1% and optimization 1%). Pulsotypes were determined based on a delineation level of 97%. We compared the obtained pulsotypes with the herd pulsotype, obtained during a previous study (Verhegghe et al., 2013a).

3.3 Statistical analysis

A chi-square test and a Fisher's exact test was used to analyze the sampling results of the carcass halves and the results of the different locations, respectively. Given that no positive samples were observed on the location "back", these data were excluded from the analysis. Analysis occurred in SPSS statistics 19 (IBM, Chicago, IL, US) and for all analyses, $P<0.05$ was considered significant.

4 Results

After direct inoculation of the initial enrichment broth, no MRSA was recovered from any carcass. After incubation of this enrichment broth, MRSA was isolated from 19 out of 40 carcass halves, i.e. 10 right halves and 9 left halves, which was not a statistically significant difference (Chi-square, $p=0.88$). In 17 cases, the strain was isolated from only one location and in two cases from two locations. From 18 halves, MRSA was only detected in the initial enrichment broth, whereas on the remaining half, MRSA was detected up till the 10^{-2} dilution of the enrichment broth. Figure V-2 shows the sampling results of the different locations. Most MRSA isolates were retrieved from the forelimb (16 carcasses). In the other cases, MRSA was isolated from the ham, abdomen, intestine cavity and sternum. When comparing the sampling results of the six sampling locations, a statistically significant difference was observed between the forelimb and the other sampling locations (Fisher's exact, $P<0.001$). No statistically significant difference was observed between the other sampling locations (Fisher's exact, $P=1.000$).

Twenty-two isolates were retrieved from 20 carcasses. All isolates were identified as MRSA CC398. Three pulsotypes were found after PFGE of which pulsotype I was retrieved from 18 out of 22 isolates (17 carcasses). The two remaining pulsotypes occurred in three (three carcasses) and one isolate (one carcass), respectively. On one carcass, pulsotypes I and II were isolated from the forelimb in different dilutions. Pulsotype I was also the only pulsotype found in the herd (Figure V-3).

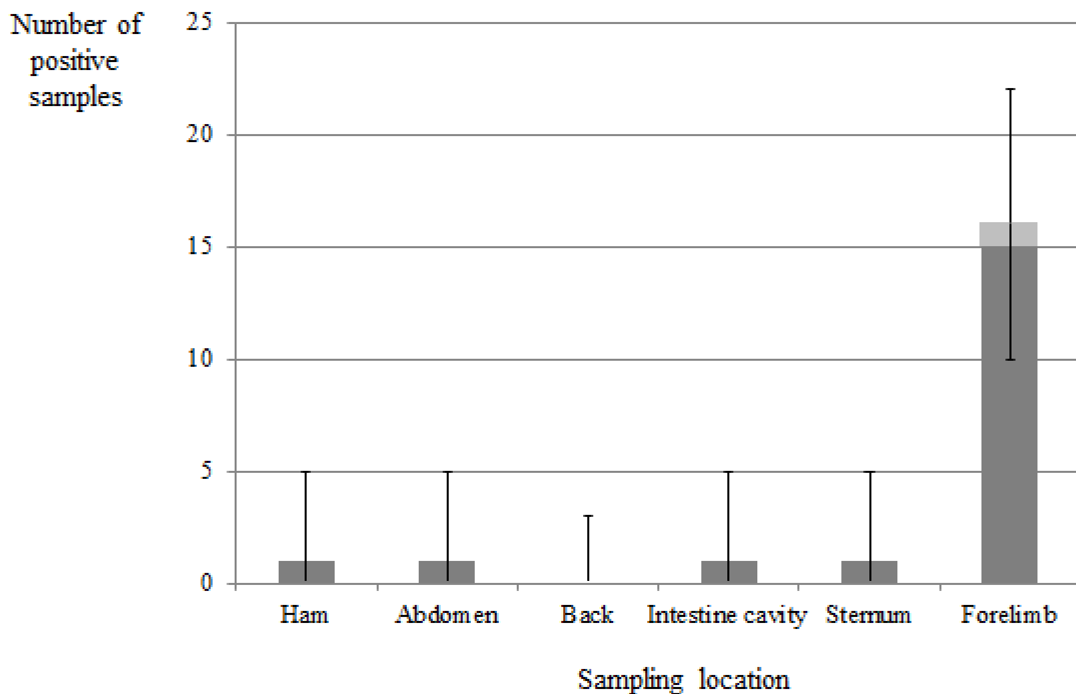

Figure V-2 Overview of the sampling results per sampling location after overnight enrichment of the samples. The results are shown in numbers with the 95% confidence intervals. In addition, the enrichment broth dilution at which MRSA detection occurred is indicated (dark gray: original enrichment broth and light grey: enrichment broth dilution 10^{-2}).

5 Discussion

Since the discovery of LA-MRSA in pigs, concerns have arisen on the transmission of this bacterium to the general human population through pork consumption. For certain zoonotic pathogens, guidelines exist for their detection on pig carcasses, which do not exist for MRSA. The use of these guidelines to determine the best sampling location for MRSA will allow the use of one sampling event for the recovery of multiple bacteria.

To date, less than 100 cfu MRSA/g pork have been reported in literature. In most pork studies, between 0 and 10% of the investigated samples were contaminated with MRSA, with and without enrichment of the samples (Van Loo et al., 2007; de Boer et al., 2009; de Jonge et al., 2010; Weese et al., 2010c). During this study, MRSA was not detected on the carcasses by direct plating. This indicates that the level of MRSA contamination on carcasses is low, which reduces the possibility of LA-MRSA exposure for the human population through meat. However, it is possible that an underestimation of the MRSA ST398 presence occurred. First, samples were taken after forced cooling of the carcasses. It is plausible that MRSA is strongly

bonded to the carcass after cooling and that the used sampling method did not retrieve all of the MRSA present. Second, cooling of the bacterium might decrease the viability of LA-MRSA strains, resulting in less detection. Third, the used isolation protocol might not be optimal for MRSA detection on carcasses. Fourth, LA-MRSA may be suppressed by other bacteria, which could result in a low or non-detection of MRSA after dilution of the sponge (Bruins et al., 2007).

After overnight incubation of the dilutions, LA-MRSA was retrieved from approximately half of the carcasses. Most MRSA was isolated from the forelimb and little MRSA was isolated from the other sampling locations tested. In the slaughterhouse, it was noticed that the bottom of the carcass, where the forelimb was located, was visually the dirtiest part of the carcass. During the present study, only one slaughterhouse and carcasses of only one MRSA-positive herd were sampled. It is uncertain whether the observed findings can be extrapolated to all slaughterhouses. Additional samplings of pig carcasses of different herds at different slaughterhouses are therefore needed to confirm the forelimb as best sampling location for MRSA. Transmission to the human population through consumption of a contaminated forelimb may be considered rather low, since forelimbs are not frequently consumed and are mostly used in stews, which has a long cooking cycle. On the other hand, handling of contaminated forelimbs by butchers or consumers may be considered as a possible risk.

Only MRSA CC398 was found on the carcasses, indicating that -in this case- no human strains contaminated the carcasses during the slaughter process. Three pulsotypes were detected of which one predominated. This pulsotype was also found in the herd where the carcasses originated from. There are some possibilities for retrieving the same strain at beginning and end of the slaughter process. When a pig is colonized with MRSA, this bacterium can be isolated from the skin, forelimbs, but also from the nares (Szabó et al., 2012; Broens et al., 2012a; Crombé et al., 2012b). The question remains whether MRSA is eliminated from the carcass during the slaughter process or not. For example, singeing of the carcass might be insufficient to decolonize the lower part of the carcass, resulting in MRSA detection on the forelimb. Besides the herd pulsotype, other pulsotypes were also found, indicating that LA-MRSA is widespread within the slaughterhouse. This would result in cross-contamination of the carcasses from, for example the environment, since it has been reported that at the end of a slaughter day MRSA ST398 was widespread in the environment of pig and broiler slaughterhouses (Mulders et al., 2010; Van Cleef et al., 2010b; Gilbert et al., 2012). Nevertheless, the carcasses of only one slaughterhouse were sampled, so, further

research is needed to investigate possible transmission routes for carcasses in a slaughterhouse. In addition, two pulsotypes were detected in different dilutions from one location, which might indicate that more than one MRSA strain is present on the carcass. Since only one suspect colony per plate was analyzed, this hypothesis still needs to be assessed.

6 Conclusion

In conclusion, during the present study performed in one slaughterhouse, it was shown that MRSA is present on carcasses in low numbers. After enrichment, the forelimb appeared the best sampling location to detect MRSA ST398 on a carcass. Moreover, the dominant pulsotype was isolated from the animals of the MRSA-positive herd and from the carcasses, indicating a transmission from the primary production. The retrieval of other pulsotypes on the carcasses implies that contamination of the carcasses from the slaughterhouse environment can also occur.

7 Acknowledgements

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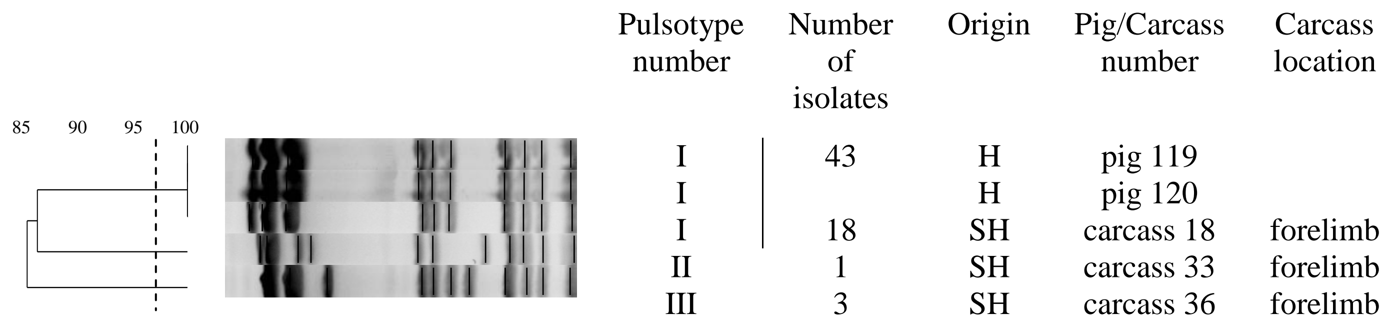

Pulsotype number	Number of isolates	Origin	Pig/Carcass number	Carcass location
I	43	H	pig 119	
I		H	pig 120	
I	18	SH	carcass 18	forelimb
II	1	SH	carcass 33	forelimb
III	3	SH	carcass 36	forelimb

Figure V-3 Comparison of the three obtained slaughterhouse pulsotypes with the herd pulsotype (two out of 43 isolates). A delineation le vel of 97% (dotted line) was applied to discriminate the different genotypes. Consecutive the dendrogram, pulsotype pattern, pulsotype number and number of isolates belonging to the pulsotype on the total number of typed isolates are shown. For each example, the origin (H: herd, SH: slaughterhouse), pig/carcass number and carcass location is given.

Chapter V.B. Prevalence and genetic diversity of livestock-associated MRSA on Belgian pork

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1 Abstract

Since the first description of livestock-associated MRSA (LA-MRSA), high isolation percentages were observed in pigs. At present, questions remain about the transmission of LA-MRSA to the general human population through pork. The objectives of the present study were to determine the prevalence of LA-MRSA on Belgian pork and to determine whether butcheries are potential LA-MRSA sources for the human population.

Meat samples (chops, bacon, minced pork, ribs, forelimbs and ears; n=137) originating from four butcheries (A to D) were collected weekly for six weeks. Twenty-five grams of chops, bacon and minced meat were 10 times diluted with salt-enriched broth. From the ribs, forelimbs and ears the cm²/sample was determined and a 1/1 dilution was performed. These original dilutions were homogenized (ribs, forlimbs and ears were removed) and a 10-fold serial dilution was made (until 10^{-6}). The dilutions were spread plated on ChromID™ MRSA plates both before and after overnight enrichment. Suspect colonies were confirmed using a MRSA-specific triplex PCR and a CC398-specific PCR. The isolates (n=147) were further characterized (SCC*mec* typing, multiple-locus variable-number tandem-repeat analysis (MLVA), antimicrobial susceptibility testing) and on a selection Pulsed Field Gel Electrophoresis (PFGE) and *spa* typing occured.

After direct plating of the dilution series, a MRSA prevalence of 8% was observed. The cfu/g or cfu/cm² ranged from 6 to 80000, respectively. After enrichment, MRSA was isolated from 98 out of 137 samples (72%). MRSA was detected in the original dilution (10^{-1}, MPN >10) of 70 samples and in dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} from 14, 5, 1, 2 and 6 samples, respectively. From the majority of rib, ear and forelimb samples, MRSA was isolated after enrichment. There was a large genetic diversity amongst the isolates within one butchery, which indicates that the obtained isolates have various sources. Twenty CC398 isolates were not resistant to any of the tested antibiotics, except β-lactam antibiotics, which is remarkable since all pig isolates are resistant to tetracyclin.

In conclusion, MRSA was found in only few pork samples after direct plating. However, large differences were seen when comparing the MRSA prevalence before and after enrichment, which indicates that enrichment is recommended for examination of MRSA on pork. The genetic diversity of the isolates indicated that a butchery can be considered as a reservoir that acts as a potential source for the general human population.

2 Introduction

In 2005, Voss and colleagues reported on a new methicillin-resistant *Staphylococcus aureus* (MRSA) type. This livestock-associated MRSA (LA-MRSA) and mainly clone MRSA ST398 (Europe) has been isolated from various livestock animals. High MRSA ST398 rates were observed in pigs, which might imply that pigs are a reservoir of MRSA. Transmission from pigs to people working with them was reported and MRSA ST398 infections in people, with and without animal contact, were occasionally described (Vanderhaeghen et al., 2010a; Weese, 2010). As a result, concerns arose about MRSA ST398 entering the food chain and subsequently infecting the general human population.

An important pathway for the transmission of micro-organisms to the general human population is meat. To date, only a limited number of studies (German, Dutch, Danish and American/Canadian) have described the presence of MRSA ST398 on pork and pork products. Low numbers of colony forming units (cfu) per gram have been reported which led to the suggestion that the transmission chance of MRSA ST398 might be rather low (Van Loo et al., 2007; de Boer et al., 2009; de Jonge et al., 2010; Weese et al., 2010b; Beneke et al., 2011; Hanson et al., 2011; Agersø et al., 2012; O'Brien et al., 2012). The aims of the present study were i) to assess the MRSA (ST398) contamination of various Belgian pork meat types and ii) to determine whether a butchery was a possible MRSA source for pork or not by collecting samples longitudinally.

3 Materials and methods

3.1 Sample collection and sample processing

Two local butcheries (butcheries A and B) and two supermarket butcheries (butcheries C and D) were randomly chosen in the region of Ghent (Belgium). Every week (six successive weeks in total), six pork samples (pork chops, bacon, minced pork meat, ribs, forelimbs and ears) were collected from each butchery (n=137). No minced pork meat was available at butchery D and on one occasion, there was no ear sample available at butchery B. After purchasing, the meat samples were directly transported to the laboratory and processed immediately upon arrival.

Twenty-five grams of chops, bacon and minced pork were diluted ten times with salt-enriched (6.5%; Sodium chloride; 1.06404; Merk, Darmstadt, DE) Mueller Hinton Broth (MHB CM0405; Oxoid, Basingstoke, UK). These dilutions were mixed mechanically in a stomacher for one minute and subsequently a ten-fold dilution series was made until dilution 10^{-5}. The ribs, forelimbs and ears were measured to determine the cm² per sample, weighted and 1/1 diluted. After homogenizing manually for one minute, the ribs, forelimbs and ears were removed and a ten-fold dilution series was made until dilution 10^{-5}. Both before and after overnight enrichment at 37°C in salt-enriched MHB, 100µl of all dilutions was spread plated onto ChromID™ MRSA (BioMerieux, Marcy l'Etoile, FR) plates. After incubation of the plates (18-20h at 37°C), the number of suspect colonies was counted. The colony forming units (cfu) per gram were calculated for chops, bacon and minced pork, whereas the cfu per cm² were calculated for the ribs, forelimb and ear. Suspect colonies were purified onto Chrom-ID™ MRSA and pure isolates were stored at -20°C in brain-heart infusion broth (BHI; CM0225; Oxoid, Basingstoke, UK) supplemented with glycerol (15% wt/vol; Fisher Scientific, Leicestershire, UK) for further analysis.

3.2 MRSA identification and MRSA ST398 confirmation

DNA was isolated from each isolate and stored at -20°C until further use (Strandén et al., 2003). Isolates were identified as MRSA as described by Maes et al., 2002. On the MRSA isolates, a CC398 specific PCR, targeting the restriction-modification system encoded by *sau*1*hsdS*1, was performed (Stegger et al., 2011).

3.3 Molecular typing

In total, 147 MRSA isolates were obtained (48 from butcher A, 40 from butcher B, 30 from butcher C and 29 from butcher D). SCC*mec* typing occurred on all isolates, based on the combination of three protocols (Oliveira and de Lencastre, 2002; Zhang et al., 2005; Milheirico et al., 2007). Multiple-locus variable-number tandem-repeat analysis (MLVA) typing was also performed on all isolates according to a modified protocol of Rasschaert et al. (2009). Briefly, the repeat region of five genes (*clf*A, *clf*B, *sdr*C, *sdr*E and SIRU21) was amplified and fragment sizing of the PCR products occurred by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems/Hitachi, Hitachinaka-shi, JP) using the GenescanTM 1200LIZ® size standard (4379950, Applied Biosystems, Warrington, UK). The obtained patterns were transformed into numeric codes using the MLVA plugin of

Bionumerics (Bionumerics version 6.5; Applied Maths, St.-Martens-Latem, BE). Categorical analysis was performed (tolerance: 0%) to cluster the different MLVA types using the unweighted pair group method using averages (UPGMA). Clustering of the predominant MLVA type of the butchery with closely related types, being single locus variants (= MLVA types with one difference in one repeat region) was performed.

From each butchery, a selection of isolates was arbitrarily chosen for Pulsed Field Gel Electrophoresis (PFGE). Thirty-six isolates (34 ST398 and two non-ST398) were selected being 10 isolates from butcher A (two forelimb, three minced pork and five rib isolates), 7 isolates of butcher B (one minced pork, one rib and five chop isolates), 13 isolates of butcher C (one chop, five forelimb isolates and seven ear isolates) and 6 isolates from butcher D (one rib and five bacon isolates). PFGE with *BstZ*I restriction (Promega, Madison, WI, US) occurred and the obtained restriction profiles were analyzed in Bionumerics 6.5 using the unweighted pair group method using averages (UPGMA) with the Dice coefficient (tolerance 1%, tolerance change 1% and optimization 1%) (Rasschaert et al., 2009). Pulsotypes were determined based on a delineation level of 97% and are given in roman numbers. *Spa* typing occurred on this selection and all non-ST398 isolates, according to the Ridom StaphType standard procedure (www.ridom.de/staphtype).

3.4 Antimicrobial susceptibility testing

The disk diffusion method according to the CLSI procedure was used to determine the antimicrobial susceptibility of all isolates (n=147) (CLSI, 2010). Neo-sensitabs (Rosco Diagnostica, Taastrup, DK) for sixteen antimicrobial agents were tested and the interpretation tables of the manufacturer (based on the CLSI method) were used. The agents were chloramphenicol (CLR; 60 µg), ciprofloxacin (CIP; 10 µg), erythromycin (ERY; 78 µg), fucidin (FUC; 100 µg), gentamicin (GEN; 40 µg), kanamycin (KAN; 100 µg), lincomycin (LIN; 19 µg), linezolid (LINE; 30 µg), mupirocin (MUP; 10 µg), quinupristin/dalfopristin (SYN; 15 µg), rifampicin (RIF; 30 µg), sulphonamides (SUL; 240 µg), tetracyclin (TET; 80 µg), tobramycin (TOB; 40 µg), trimethoprim (TRI; 5.2 µg) and tylosin (TYL; 150 µg). *S. aureus* ATCC 25923 and a MRSA ST398 strain (MB4360) were used as reference strains. From 24 CC398 tetracyclin sensitive isolates, the presence of the tetracycline resistance genes *tet*M and *tet*K was assessed by a PCR as described by Ng et al. (2001).

4 Results

After direct plating of the pork homogenate, MRSA was detected in 11 out of 137 (8%) meat samples being one bacon (butcher A), one rib (butcher B), two chops (butchers A and D), three forelimbs (butchers A, B and C) and four ear samples (butchers A, B and C). The cfu ranged from 200 to 80000 per gram and from 6 to 14776 per cm² (Table V-1). After enrichment, MRSA was isolated from 98 samples (72%) being 23/23 (100%) samples, 21/24 (88%) forelimbs, 20/24 (83%) ribs, 11/18 (61%) minced pork, 12/24 (50%) bacon and 11/24 (46%) chop samples. After enrichment, the original dilution (10^{-1}, most probable number (MPN) >10) was positive for 70 samples (10 chops, 9 bacon, 10 minced meat, 15 rib, 15 forelimb and 11 ear samples). Fourteen samples (one bacon, three rib, four forelimb and six ear samples) were MRSA positive after enrichment up to dilution 10^{-2} (MPN>100) and five samples (one bacon, one rib, one forelimb and two ear samples) up to dilution 10^{-3} (MPN>1000). MRSA was detected after enrichment from one chop sample until dilution 10^{-4} (MPN>10000) and one rib and ear sample until dilution 10^{-5} (MPN>100000). MRSA was found in six samples (one bacon, one mince meat, one forelimb and three ear samples) until dilution 10^{-6} (MPN> 1000000) (Table V-1). From eight out of 11 samples, where MRSA was isolated after direct plating, MRSA was also detected after enrichment (Table V-1).

From the 147 MRSA isolates, 143 (97%) belonged to CC398. Four SCC*mec* cassette types were detected in the MRSA ST398 isolates: SCC*mec* type V (111/143), IVa (28/143), IV (1/143) or a non-typeable cassette type 3 (2/143, *mec*A complex NT/*ccr* complec C) (Table V-2). The non-CC398 isolates carried SCC*mec* type IV (2/4), IVa (1/4) or V (1/4). In total, forty-three MLVA types were isolated of which 21, 15, 15 and 13 types were observed in butcheries A to D, respectively. Clustering of the types resulted in two clusters in butcheries A, B and D and three clusters in butchery C (Table V-2). Two clusters (I and II) were found in all butcheries. Twenty percent of the strains (all MRSA ST398) belonged to the dominant MLVA type (MLVA code: 33-56-36-7-3). These isolates originated from various meat samples and from different sampling events. Moreover, isolates originating from the meat samples at one sampling event or from the one meat type at various sampling events did not always belong to the same MLVA cluster/type.

Table V-1 Results of the MRSA detection in the different pork meat types on six sampling occasions after direct plating (DP) or enrichment (E). The result of the direct plating are shown in cfu/g for the pork chops and bacon, whereas cgu/cm² for the ribs, forelimbs and ears. For the enrichment, the highest dilution at which MRSA was found, is indicated (-: MRSA negative, NA: Not available).

		Sampling event											
		T1		T2		T3		T4		T5		T6	
Butcher	Meat	DP	E	DP	E	DP	E	DP	E	DP	E	DP	E
A	Chops	-	-	-	-	8100	-	-	-	-	10^{-1}	-	-
	Bacon	-	10^{-2}	-	-	200	-	-	-	-	10^{-6}	-	-
	Minced meat	-	10^{-6}	-	-	-	-	-	-	-	10^{-1}	-	-
	Rib	-	10^{-3}	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	10^{-1}
	Forelimb	3557	10^{-6}	-	10^{-2}	-	10^{-1}	-	10^{-1}	-	-	-	10^{-1}
	Ear	-	10^{-6}	-	10^{-2}	541	10^{-5}	-	10^{-1}	-	10^{-1}	-	10^{-2}
B	Chops	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	-	-	10^{-1}	-	10^{-1}
	Bacon	-	-	-	10^{-1}	-	10^{-3}	-	10^{-1}	-	-	-	-
	Minced meat	-	-	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	10^{-1}
	Rib	-	10^{-2}	-	10^{-1}	930	10^{-1}	-	10^{-2}	-	10^{-1}	-	10^{-1}
	Forelimb	-	10^{-1}	-	10^{-2}	6704	10^{-3}	-	10^{-2}	-	10^{-1}	-	10^{-1}
	Ear	14776	10^{-6}	-	10^{-1}	184	10^{-3}	-	10^{-2}	-	10^{-2}	NA	NA
C	Chops	-	10^{-4}	-	-	-	10^{-1}	-	-	-	-	-	-
	Bacon	-	-	-	10^{-1}	-	10^{-1}	-	-	-	-	-	-
	Minced meat	-	10^{-1}	-	10^{-1}	-	-	-	10^{-1}	-	10^{-1}	-	-
	Rib	-	-	-	-	-	10^{-1}	-	10^{-1}	-	-	-	-
	Forelimb	-	10^{-1}	-	10^{-1}	-	10^{-1}	6	10^{-1}	-	10^{-1}	-	10^{-1}
	Ear	-	10^{-1}	-	10^{-1}	-	10^{-3}	6	10^{-6}	-	10^{-1}	-	10^{-1}
D	Chops	-	10^{-1}	-	10^{-1}	-	-	80000	-	-	-	-	10^{-1}
	Bacon	-	10^{-1}	-	10^{-1}	-	10^{-1}	-	-	-	10^{-1}	-	10^{-1}
	Rib	-	10^{-1}	-	10^{-1}	-	10^{-5}	-	10^{-2}	-	10^{-1}	-	10^{-1}
	Forelimb	-	10^{-1}	-	10^{-1}	-	10^{-2}	-	-	-	10^{-1}	-	-
	Ear	-	10^{-2}	-	10^{-1}	-	10^{-2}	-	10^{-1}	-	10^{-1}	-	10^{-1}

Table V-2 Overview of the MRSA ST398 isolates, ordered according to the MLVA results. For each MLVA cluster, the dominant MLVA type is given between brackets. MLVA types consisting of only one isolate are grouped in the category singleton. For each MLVA cluster/type the butchery, meat sample (Ba: bacon, C: chops, E: ear, Fo: forelimb, Mm: minced meat, R: rib), sampling event (time point 1 to 6), SCC*mec* type (NT: *mec*A complex NT/*ccr* complec C), *spa* type (when determined), pulsotype (when determined) and antibiotic resistance profile are shown. The numbers between brackets indicate the number of isolates.

MLVA cluster/type	Butchery	Meat sample (number of isolates)	Sampling event	SCC*mec*	*Spa* type (number of isolates)	Pulsotype (number of isolates)	AB resistance profile
I (33-56-36-7-3)	A	C(2), Ba(1), Mm(1), R(1), Fo(2), E(1)	1	IVa	t011(3)	V(3)	Gen, Kan, Tet, Tob, Tri
		R(1), E(1), Fo(1)	6	V	t011(1)	VIII(1)	Cip, Tet, Tri
		Fo(2)	1,4	V			Ery, Linco, Tet, Tri, Tyl
		Ba(1)	5	V			Linco, Syn, Tet
		E(1)	4	V			Tet
		Fo(1)	3	V			Cip, Linco, Syn, Tet, Tri
	B	E(2), C(1), Fo(1)	3, 4, 5(2)	V	t011(1)	VIII(1)	Cip, Tet, Tri
		Fo(1), Ba(1)	1, 4	V			Linco, Tet, Tri
		C(1), Ba(1)	3	V	t011	VIII	Cip, Linco, Tet, Tri
		Mm(1), E(1)	1, 5	IVa			Gen, Kan, Tet, Tob, Tri
		Mm(1), E(1)	4, 5	V			Cip, Ery, Linco, Tet, Tri, Tyl
		Fo(1)	4	V			Tet
		C(1)	1	V			Tet, Tri
		Fo(1)	6	IVa			Gen, Kan, Tet, Tob, Tri, Tyl
		R(1), Fo(1)	4, 6	IVa			Ery, Gen, Kan, Linco, Tet, Tob, Tri, Tyl
	C	C(1), Fo(1), E(1)	3	V	t011 (2)	VIII(1), XI(1)	Cip, Linco, Tet, Tri
		Ba(1), E(1)	3	V	t011(1)	VIII(1)	Cip, Linco, Syn, Tet, Tri
		Fo(1)	1	V	t011	VIII	Tet, Tri
		Fo(1)	4	V	t011	VIII	Cip, Tet
		E(1)	6	V	t011	VIII	Cip, Tet, Tri
		Fo(1)	5	V	t3423	VIII	Linco, Syn, Tet
		E(1)	4	IVa	t011	V	Gen, Kan, Tet, Tob, Tri
		R(1)	3	V			Cip, Ery, Linco, Tet, Tri
		E(1)	5	V	t011	VIII	Cip, Ery, Linco, Tet, Tri, Tyl
	D	Ba(1), E(1)	6	V	t011(1)	VIII(1)	Tet, Tri
		C(1), Fo(1)	1, 5	V			Cip, Tet
		Fo(1), E(1)	3, 4	V			Cip, Linco, Tet, Tri
		R(2)	4,6	V		VI(1)	Ery, Linco, Tet, Tob, Tri, Tyl
		E(1)	1	IVa			Tet, Tob, Tri
		E(1)	2	V			Cip, Tet, Tri
		R(1)	3	V			Cip, Linco, Syn, Tet, Tri

MLVA cluster/type	Butchery	Meat sample (number of isolates)	Sampling event	SCC*mec*	*Spa* type (number of isolates)	Pulsotype (number of isolates)	AB resistance profile
II	A	Ba(1), R(1)	2, 3	V	t011(1)	IX(1)	Tet, Tri
(33-57-37-7-3)	B	Mm(1)	2	V			Linco, Tet
		E(1)	2	V			Cip, Tet, Tri
		Fo(1)	3	V			Linco, Tet, Tri
		Fo(1)	2	V			Cip, Ery, Linco, Tet, Tri, Tyl
	C	Ba(1), E(1)	2	V	t011(1)	VIII(1)	Ery, Linco, Syn, Tet, Tri, Tyl
		E(1)	6	V	t011	VIII	Cip, Tet, Tri
		E(1)	1	V	t011	VII	Ery, Linco, Tet
		Fo(1)	2	V	t011		Linco, Tet, Tri
	D	C(1), Ba(1), R(1), Fo(1)	2	V	t011(1)	X(1)	Tet, Tri
		Ba(1), C(1), E(1)	2, 5, 6	V	t011(1)	VIII(1)	Cip, Tet, Tri
		Ba(1)	1	V	t011	VIII	Cip, Tet
		R(1)	6	V			Ery, Linco, Tet, Tob, Tri, Tyl
III (34-61-34-7-3)	C	E(6), R(1)	3, 4(1)	V	t011(1)	IV(1)	Cip, Tet, Tri
Non-typeable	A	C(5), Ba(2), Mm(5), R(1)	3	V	t011(1)	II(1)	None
	B	R(1)	3	V			Tet
		Fo(4), E(3)	3	V			None
	D	C(2), Ba(1)	4	V			Mup
34-57-36-7-3	A	C(1)	1	IVa			Gen, Kan, Tet, Tob, Tri
	B	E(1)	1	IVa			Gen, Kan, Tet, Tob, Tri
		R(2)	3, 5	V	t011(1)	V(1)	Ery, Gen, Kan, Linco, Tet, Tob, Tri, Tyl
	C	Fo(1)	6	IVa	t011	VIII	Gen, Kan, Tet, Tob, Tri
	D	Fo(2)	1	IVa			Tet, Tob, Tri
32-56-37-38-4	B	O(1)	1	V			Clr, Cip, Linco, Syn, Tet, Tri
	C	C(1)	1	V			Clr, Cip, Linco, Syn, Tet
		Mm(1)	4	V			Clr, Cip, Linco, Tet, Tri
33-50-36-23-4	A	C(1), E(1)	5	NT			Ery, Linco, Tet, Tyl
32-58-36-7-3	A	E(2)	3	V			Tet
33-57-37-38-4	C	Mm(1)	2	V			Clr, Cip, Linco, Syn, Tet, Tyl
		Mm(1)	5	V			Mup

MLVA cluster/type	Butchery	Meat sample (number of isolates)	Sampling event	SCC*mec*	*Spa* type (number of isolates)	Pulsotype (number of isolates)	AB resistance profile
Singletons[a]	A	E(1), Fo(1)	2	IVa			Gen, Kan, Tet, Tob, Tri
		R(3)	2	V	t011(1)	VIII(1)	Cip, Ery, Linco, Tet, Tri, Tyl
			4	V	t034	V	Clr, Cip, Linco, Syn, Tet, Tri
			5	NT	t034		Ery, Linco, Tet, Tyl
		E(1)	1	IVa			Ery, Gen, Kan, Linco, Tet, Tob, Tri, Tyl
		E(2)	3	V			Tet
		Fo(2)	1	V	t2370(1)	VIII(1)	Ery, Linco, Tet, Tri, Tyl
	B	C(1)	2	IVa	t011(1)	III(1)	Gen, Kan, Tet, Tob, Tri
		C(1)	6	V	t011	VIII	Linco,Tet
		R(1), E(1)	1	V			Tet, Tri
		R(1)	2	IV			Ery, Gen, Kan, Linco, Tet, Tob, Tri, Tyl
	C	Mm(1)	1	V			Cip, Ery, Linco, Tet, Tri, Tyl
		Mm(1)	5	V			Clr, Cip, Gen, Kan, Linco, Syn,; Tet, Tob
		E(2)	3	V			Cip, Tet, Tri
	D	Ba(1), R(1)	1, 3	V	t011(1)	VIII(1)	Cip, Ery, Linco, Tet, Tri, Tyl
		R(1)	5	V			Tet, Tri
		E(2)	3, 5	IVa			Gen, Kan, Tet, Tob, Tri

[a] All isolates belonging to a singleton are grouped per butchery according to similar features and not the numeric MLVA code

Table V-3 Overview of the different antimicrobial susceptibility profiles and the number of ST398 and non ST398 MRSA isolates belonging to the profile (Clr: Chloramphenicol, Cip: Ciprofloxacin, Ery: Erythromycin, Gen: Gentamicin, Kan: Kanamycin, Linco: Lincomycin, Mup: Mupirocin, Syn: Quinu/dalfopristin, Tet: Tetracyclin, Tob: Tobramycin, Tri: Trimethoprim, Tyl: Tylosin).

	Number of isolates belonging to the profile		
Antimicrobial susceptibility profile	ST398 isolates	non ST398 isolates	Total
None	20	2	22
Cip, Tet, Tri	19	0	19
Gen, Kan, Tet, Tob, Tri	19	0	19
Tet, Tri	13	0	13
Cip, Ery, Linco, Tet, Tri, Tyl	8	0	8
Cip, Linco, Tet, Tri	7	0	7
Tet	7	0	7
Ery, Gen, Kan, Linco, Tet, Tob, Tri, Tyl	6	0	6
Cip, Tet	4	0	4
Cip, Linco, Syn, Tet, Tri	4	0	4
Ery, Linco, Tet, Tri, Tyl	4	0	4
Linco, Tet, Tri	4	0	4
Mup	4	0	4
Ery, Linco, Tet, Tyl	3	0	3
Tet, Tob, Tri	3	0	3
Clr, Cip, Linco, Syn, Tet, Tri	2	0	2
Ery, Linco, Syn, Tet, Tri, Tyl	2	0	2
Ery, Linco, Tet, Tob, Tri, Tyl	2	0	2
Linco, Tet	2	0	2
Linco, Syn, Tet	2	0	2
Cip, Gen, Tet, Tri	1	0	1
Cip, Ery, Linco, Tet, Tri	1	0	1
Clr, Cip, Linco, Tet, Tri	1	0	1
Clr, Cip, Linco, Syn, Tet	1	0	1
Clr, Cip, Linco, Syn, Tet, Tyl	1	0	1
Clr, Cip, Gen, Kan, Linco, Syn, Tet, Tob	1	0	1
Ery, Linco, Tet	1	0	1
Ery, Fuc, Linco, Tob, Tri	0	1	1
Ery, Kan, Linco, Tet, Tob, Tri, Tyl	0	1	1
Gen, Kan, Tet, Tob, Tri, Tyl	1	0	1

The 36 isolates, selected for PFGE, were divided into 12 pulsotypes of which type VIII predominated in 18 isolates (50%). Within CC398 (n=34), four *spa* types were found: t011 (88%), t034 (6%), t2370 (3%) and t3423 (3%). Within non-CC398 (n=4), *spa* type t127 was detected in two isolates, whereas *spa* type t011 in one isolate. One non-CC398 isolate was non-typeable with *spa* typing (Table V-2).

Antimicrobial susceptibility testing revealed 30 antibiotypes (Table V-3). Antibiotypes Cip-Tet-Tri and Gen-Kan-Tet-Tob-Tri were found in 19 MRSA isolates each (13%) and antibiotype Tet-Tri in 13 isolates (9%). The remaining profiles were found in seven or less isolates (Table V-2 and V-3). Twenty out of 147 MRSA isolates (15%) were not resistant to any of the tested antibiotics although these 20 isolates all carried the *tet*M and *tet*K gene.

5 Discussion

The increased presence of MRSA ST398 in pigs worldwide, gave rise to concerns about the role of pork in the transmission of this potential pathogen to the human population. Handling contaminated meat by butchers or consumers may be considered as a possible risk for contracting MRSA. Cooking or heating up the meat will result in killing MRSA. Still, caution is needed to prevent the spread of MRSA throughout the household after meat handling (Nunan and Young, 2007). Meat is known as an important vector in the transmission of zoonotic bacteria, but little is known about MRSA ST398. To our knowledge, this is the first Belgian study on the pork MRSA ST398 presence.

At present, only three studies determined the MRSA cfu/g pork (de Boer et al., 2009; de Jonge et al., 2010; Weese et al., 2010b). In comparison to these studies where between 0.06 and <100 cfu/g pork were detected, the colony counts of the present study were remarkably higher (between 200 and 80,000 cfu/g). Both Dutch studies used an enrichment step before estimating the MPN of cfu/g. Besides the different protocols used, a possible explanation for the observed differences might be the pork types that were analyzed. The two Dutch studies did not indicate which pork type was analyzed, whereas the Canadian study only analyzed pork chops and minced meat. Another possibility might be that the meat samples, analyzed during the present study, were more contaminated with MRSA. Only one study reported on the prevalence of a pork type individually. Kelman and colleagues (2011) found a MRSA prevalence of 0.3% in American minced pork after direct plating, whereas no MRSA was isolated from minced pork by direct plating during the present study. High colony counts

were detected on chops, forelimb and ear samples. Compared to forelimb and ear samples, chops are often consumed.

Several studies determined the MRSA prevalence of pork after enrichment of the samples, which resulted in an European pork prevalence between 0% (Switzerland) and 11% (The Netherlands) and an American/Canadian pork prevalence lower than 10% (de Boer et al., 2009; Huber et al., 2010; Weese et al., 2010b). The overall prevalence of the present study (67%) is considerably higher than the other studies and differences in prevalence were observed between the pork types. The prevalence of each pork type was higher than the European and American-Canadian studies. For chops, a prevalence of 46% was observed in comparison to 5% (US), 6% (The Netherlands), 6% (US) and 14% (Canada) (Weese et al., 2010b; Beneke et al., 2011; O'Brien et al., 2012; Jackson et al., 2013). In 61% of the minced pork samples, MRSA was isolated, whereas in Canada from 6.3% and in the US from 9% of the minced meat samples (Weese et al., 2010b; O'Brien et al., 2012). MRSA was detected on 83% of the rib samples in comparison to 8% and 9% in the US (O'Brien et al., 2012; Jackson et al., 2013). Beneke et al. (2011) collected meat samples at the slaughterhouse, whereas Weese et al. (2010c), O'Brien et al. (2012) and Jackson et al.(2013) collected meat at retail level. During the present study, pork was purchased in butcheries, where meat was processed on site. Beneke et al. (2011) purchased meat at the slaughterhouse, whereas the other three studies did not specify whether meat was processed on site or not. It is possible that meat is contaminated upon meat transport or meat handling, but this still needs to be assessed.

Still, caution is needed when interpreting and comparing prevalences between studies since different sample processing protocols were used among the studies. A first important difference between the studies is the enrichment step. The present study, which yielded the highest MRSA percentages, used a one-step enrichment in 6.5% salt-enriched broth. The American and Canadian studies used a 7.5% salt and mannitol enriched broth and the American study a broth supplemented with tellurite. Beneke and colleagues (2011) used a two-step enrichment: enrichment in a salt-enriched broth, followed by an enrichment in a antibiotic-enriched broth. It remains possible that not all enrichments/enrichment steps are equally beneficial for MRSA (CC398) isolation, resulting in an underestimation of the MRSA CC398 presence on meat. A preliminary study, performed at our laboratory, indicated that the use of a two-step enrichment of which one step in an antibiotic enriched broth did not result in higher MRSA isolation. On the contrary, less MRSA was found after enrichment in antibiotic enriched broth (data not shown). In addition, during the present study, ribs, forelimbs and ears

were diluted with the salt-enriched broth, whereas both the American and Canadian studies used a peptone broth to rinse ribs and others, which was diluted afterwards, which may have an influence on the MRSA detection (Weese et al., 2010c; O'Brien et al., 2012; Jackson et al., 2013). Moreover, during this study, differences were observed between the MRSA isolation results before and after enrichment. This finding also indicates that the used direct plating and enrichment procedure did not yield all MRSA present on the meat samples. Another difference between the studies was the use of various chromogenic media for MRSA detection. Perhaps not all chromogenic media yield a good performance for the detection of LA-MRSA from meat samples, as was seen for nasal samples (Graveland et al., 2009; Pletinckx et al., 2012). These differences indicate that further research on this processing protocol is needed.

The majority of the obtained isolates belonged to CC398, the clonal complex of LA-MRSA. This was expected since in most recent European studies, MRSA ST398 was isolated from pork (de Boer et al., 2009; Beneke et al., 2011; Agersø et al., 2012). Four non-CC398 were isolated. One of these isolates belonged to *spa* type t011, which is a *spa* type that was only associated with MRSA ST398 before. A possibility is that a false-negative result was obtained during the CC398 PCR or that *spa* type t011 can be associated with other CCs, which needs confirmation by performing multilocus sequence typing. *Spa* type t127 was associated with ST1, a human MRSA type (http://spaserver.ridom.de).
Longitudinal sampling was performed to determine whether the butchery was a MRSA source or not. Molecular typing revealed a genetic divers population of isolates within one butchery and one pork type. An explanation for this large genetic diversity is that in a butchery various isolates, originating from carcasses and meat are gathered. This obtained population appears to maintain itself, resulting in the transmission of these isolates and subsequently in the observed diversity. Therefore, a butchery can be considered as a reservoir that acts as a potential source for the general human population.

Three MLVA types were detected in all butcheries and the majority of these isolates also belonged to the same pulsotype. This may be an indication of a common source for these isolates. It is possible that the four butchers received pork from the same slaughterhouse or same meat distributor, but no information on this is available. Another possibility is that these isolates originate from the farm and maintain throughout the pig production chain. When comparing the present MLVA and pulsotypes with isolates obtained during previous samplings at the farm and slaughterhouse, the same pulsotypes and MLVA types were

detected as seen in pigs (Verhegghe et al., 2011, 2013a and b). It has already been suggested by Verhegghe and coworkers (2011, 2013a and b) that few MRSA ST398 strains may be widespread within the pig population, resulting in a constant flow-through to slaughterhouses, workers, transport trucks and butcheries. These results indicate that "one" MRSA strain is widespread throughout the pork production chain, but this still needs confirmation. Subsequently, control measures can be created to reduce this MRSA source, for example decolonization of the animals at farm level, thorough disinfection of slaughterhouses and butcheries, good hand hygiene of slaughterhouse workers and butchers and good conservation of the meat to reduce bacterial growth.

Besides various genotypes, a lot of antibiotypes were detected and the observed antimicrobial variety was similar to the resistance of pig isolates (Verhegghe et al., 2012). In general, CC398 isolates are resistant to tetracyclines and nearly all isolates are resistant to trimethoprim (Vanderhaeghen et al., 2010a). However, during the present study, less isolates were resistant to these antimicrobials: approximately 80% and 70% for tetracycline and trimethoprim, respectively. Twenty MRSA CC398 isolates were sensitive to all antimicrobial agents tested, except β-lactam antibiotics, which was quite unexpected. One possibility is that pig CC398 isolates persist and grow throughout the food chain and subsequently colonize meat. Finding antimicrobial sensitive CC398 isolates indicates that these isolates can lose their antibimicrobial resistance when moving through the food chain as was seen in CA-MRSA strains (Chambers, 2001). The 20 isolates still carried the tetracyclin resistance genes *tet*M and *tet*K. It is possible that the resistance genes are suppressed or expressed at a lower level. As only one antimicrobial concentration was tested, this might result in the observed sensitivity. Remarkably, these isolates did not generate PCR amplicons with MLVA typing and originated from butcheries A and B. Four other isolates, also non-typeable with MLVA, were only resistant to tetracycline (one isolate from butcher A) and mupirocine (three isolates from butcher D). Additional research is needed on these isolates.

6 Conclusion

In conclusion, the MRSA prevalence on Belgian pork samples was 8% after direct plating, with the highest isolation rates on chops, ears and forelimbs. Higher isolation percentages (72%) were observed after enrichment. The pork isolates appeared less resistant to tetracyclin and trimethoprim compared to pig isolates. This is the first description of MRSA CC398 isolates susceptible to all tested antimicrobials, except β-lactams. These isolates were tetracycline susceptible, although the tetracycline resistance genes were present. Due to the genetic diversity of the isolates, the butchery is considered as a reservoir, where various MRSA isolates are gathered and persist. This reservoir can be considered as a possible MRSA source for the general human population.

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Chapter VI. General discussion

The detection of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in the pig population, revealed an unknown MRSA source for the human population in these animals (Voss et al., 2005). Pigs are considered to be mainly carriers of LA-MRSA, since only few MRSA infections have been reported in this animal species. One major LA-MRSA clone is MRSA ST398. Description of MRSA ST398 infections in humans, mainly related to contact with pigs, led to concerns about the transmissibility of this bacterium from pig farms and pork to the general human population.

The scope of this PhD was to investigate the molecular epidemiology of LA-MRSA throughout the Belgian pig production sector and included sampling events at three stages in the pork production chain, being at farm level, at slaughterhouse level and at meat level. In such way, we retrieved important information about the presence and genetic diversity of LA-MRSA in this chain. An essential observation is the presence of LA-MRSA throughout the various stages. At farm level, approximately 60 to 80% of the pigs are MRSA carriers at slaughter. Pig farming in Flanders represents approximately 94% of the Belgian pig farming (Platteau et al., 2012). Even though a low number of pig farms were sampled, this means that pigs represents a large reservoir of LA-MRSA for the pork production chain. In addition, considering that approximately 80% of the pigs, entering the slaughterhouse are MRSA carriers, it is not so surprising that LA-MRSA was found on approximately half of the carcasses. However, these results should be considered as an indication of the MRSA presence on carcasses, since only 40 carcasses of one herd at one slaughterhouse were sampled. After analyzing meat samples, we could also isolate MRSA from a large number of samples (8% of the samples without enrichment and 72% of the samples after enrichment). Taking these results into consideration brings up some important questions which will be discussed.

What did we learn from the three studied stages of the pork production chain?

The statistical analysis of the longitudinal study showed that the factor **farm** had a significant effect on the MRSA status of the piglets. This was not unexpected: a farm and more specific a farrow-to-finish farm can be considered as a (more or less) closed but dynamic system in which the animals reside within connected compartments and are under the influence of farm specific factors. Three factors played an important role in the colonization of piglets: the mother sow, the piglets themselves and the environment. Statistical analysis revealed an influence of the MRSA status of the mother sow at farrowing on the piglet's MRSA status.

When a sow is a MRSA carrier, MRSA may be isolated from the nares, skin, but also from the vagina (Moodley et al., 2011a). Upon birth, initial contact with the mother sow can result in the first colonization of piglets: we found piglets carrying the same genotypes (but not always) as their mother sows. In addition, molecular typing revealed the spread of some genotypes throughout the piglet group. This confirms the fast transmission of LA-MRSA between piglets (Broens et al., 2012b; Crombé et al., 2012b). From the present work, it remains unclear what the exact role of environmental contamination is. To confirm these results and retrieve more information about the transmission of LA-MRSA within a farm, it could be interesting to focus more deeply on the transmission between a sow, her offspring and environmental factors of the farm. Possibilities are: more intensive sampling events of the animals throughout the nursing unit, studying animals from different sow groups, comparison of the genotypes and using controlled conditions as used in transmission experiments (Broens et al., 2012b; Crombé et al., 2012b; Gibbons et al., 2013).

Here, we described two colonization trends: a low colonization trend (MRSA was isolated only sporadically from the pigs in the nursing unit and an increase in MRSA isolation was observed at the end of the growing period) and a high colonization trend (MRSA was isolated from the majority of pigs from farrowing until slaughter age). When comparing these colonization trends with the colonization trends reported in literature (Nathaus et al., 2010; Weese et al., 2010a; Broens et al., 2012b), the question arises whether these other trends (such as low colonization percentages throughout the lifespan or an increase at the beginning of the growing period followed with a decrease at the start of the finishing period) are also present on Belgian pig farms. Moreover, it should be investigated whether the low colonization trend might be a transition state to a high colonization trend. Resampling the farms might elucidate this hypothesis.

The four farmers were subjected to a questionnaire about the farm management. Even though only four farms were sampled and, therefore, no risk factor analysis could be performed, some differences between the low (farms A and B) and high (farms C and D) colonization farms could be indicative and should be further investigated: i) use of a fixed route throughout the farm as seen on the low colonization farms; ii) effect of an “empty and clean” period between 7 and 10 days, as practiced in a three weeks production cycle (farms A and B). It has been reported that an “empty and clean” period of less than 6 days is a known risk factor for *Salmonella* (Fosse et al., 2009); iii) antimicrobial treatment of sows after farrowing (A: lincomycin and B: penicillin) or no treatment (C and D); iv) antimicrobial treatment of the

pigs: farmers A and B treated all animals in the growing unit with colistin/amoxicillin and trimethoprim/sulfadiazine, respectively, whereas on farms C and D, the piglets were treated in the nursery unit with ampicillin and afterwards in the growing unit with colistin/amoxicillin and ampicillin, respectively; v) presence of an additional animal species (farms C and D are pig-poultry farms); vi) distance to other pig farms (within 1km of farms C and D) and vii) the use of cleaning and disinfection strategies (no disinfection on farms A and B).

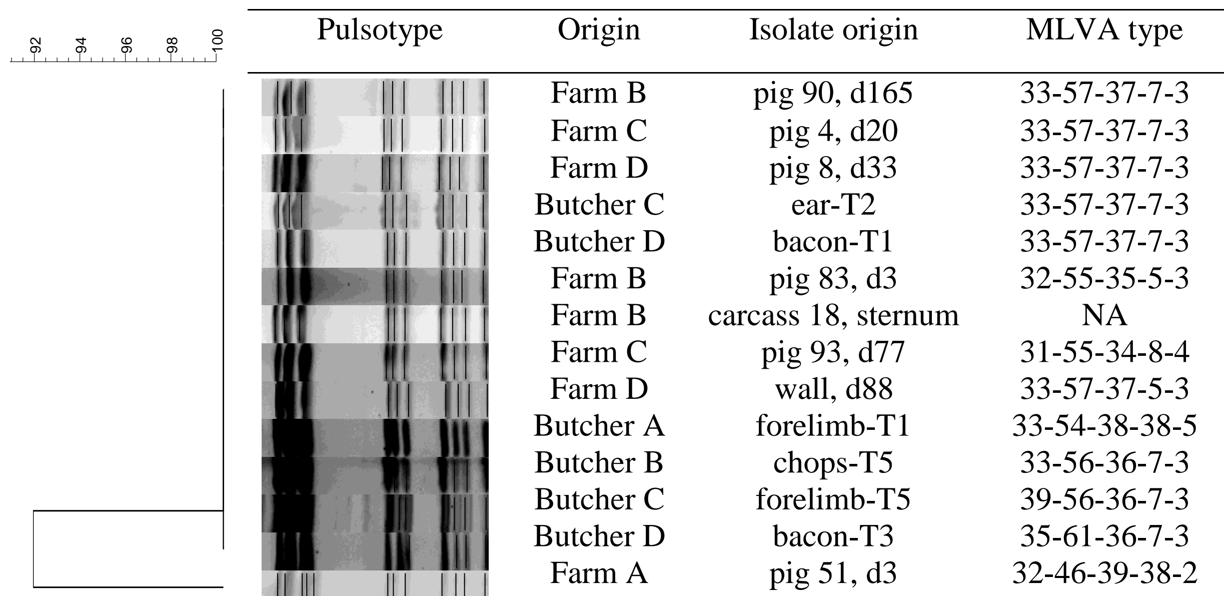
We showed that the best sampling location on a **pig carcass** was the forelimb. This correlates with the lower part of the carcass being visually dirtier than the upper part of the carcass. In addition, it appeared that the sampling scheme used for *Salmonella* detection was also useful to determine the presence of MRSA on the carcass, but other sampling shemes have not been evaluated. Still, carcasses from more than one slaughterhouse should be sampled. From 8% of the **pork meat** samples (72% after enrichment), MRSA ST398 was isolated, which was quite high compared to results published by other research groups (see section 2.6.5; de Boer et al., 2009; de Jonge et al., 2010; Weese et al., 2010b; O'brien et al., 2012). This can be attributed to different protocols for sampling, processing and MRSA isolation used by different research groups. For example, for meat samples, different enrichment steps have been used: we used a one-step enrichment in 6.5% salt-enriched broth, Weese and coworkers (2010b) used a one-step enrichment in 7.5% salt + mannitol and other studies used a two-step enrichment in 6.5% or 7.5% salt-enriched broth and subsequently in phenol-red solution enriched with aztreonam + ceftizoxime (de Boer et al., 2009; de Jonge et al., 2010, respectively). A preliminary study at our lab (data not shown) indicated that an additional enrichment step in antibiotic-supplemented (aztreonam + ceftizoxime) broth, as described by de Boer et al. (2009) did not result in additional MRSA isolation compared to only salt-enrichment. Our one-step enrichment yielded even higher MRSA isolation rates from pork compared to the studies of de Jonge et al. (2010) and Weese et al. (2010b). This demonstrates the need to study the effect and efficacy of the various enrichment methods on the diverse matrices. Creating one standard protocol would also allow better comparison between the sampling results.

An important consideration of our studies is that few farms, slaughterhouses and butcheries were included in the studies. Another approach could have been sampling more entities of the pork production chain. For example, four pig farms (sampled during the longitudinal study) are not a good representative for the 5400 pig farms, present in Flanders. However, when increasing the number of farms, it should be considered whether it is still possible to sample the same number of pigs on each sampling event. In such way, the present work could serve

as an indication for future sampling events. In addition, the present approach allowed us to subject a large subset of isolates, originating from one entity, to a thorough molecular typing, allowing to assess the relevance of MLVA typing and to compare the genetic diversity of isolates originating from various stages of the pork production chain.

Throughout the studies, various molecular typing methods were used to gain more insight into the genetic variability of the obtained isolates, but what did we learn from this typing?

The main observation was the identification of one apparent dominant LA-MRSA clone in the pork production chain: the same LA-MRSA clone was found on the farms (B, C and D), in the slaughterhouse and on the pork samples (Figure VI-1). This indicates that there might be a high level of exchange of strains between farms and that there is a flow-through from birth to slaughter, with the sows as initial source for the pigets. However, on farm A, a different LA-MRSA strain was present. Seen the diverse locations of the farms (A and B: West-Flanders, C: East-Flanders and D: Antwerp), the slaughterhouse (East-Flanders) and the butcheries (East-Flanders), Flanders might be considered as one large regional unit with the dispersion of a few clones. Additional samplings and molecular typing on the various stages in the production chain and more geographical locations will be necessary to confirm these hypotheses.



Pulsotype	Origin	Isolate origin	MLVA type
	Farm B	pig 90, d165	33-57-37-7-3
	Farm C	pig 4, d20	33-57-37-7-3
	Farm D	pig 8, d33	33-57-37-7-3
	Butcher C	ear-T2	33-57-37-7-3
	Butcher D	bacon-T1	33-57-37-7-3
	Farm B	pig 83, d3	32-55-35-5-3
	Farm B	carcass 18, sternum	NA
	Farm C	pig 93, d77	31-55-34-8-4
	Farm D	wall, d88	33-57-37-5-3
	Butcher A	forelimb-T1	33-54-38-38-5
	Butcher B	chops-T5	33-56-36-7-3
	Butcher C	forelimb-T5	39-56-36-7-3
	Butcher D	bacon-T3	35-61-36-7-3
	Farm A	pig 51, d3	32-46-39-38-2

Figure VI-1 Dendrogram containing the dominant pulsotypes obtained on the four longitudinally sampled farms, the pig carcasses and pork samples of the four butchers. Per pulsotype, the results of two isolates are shown as an example. For each sample, the origin, isolate origin (d: days after farrowing, T: sampling event) and MLVA type are shown. No MLVA typing occurred on the carcass isolate (NA).

We used MLVA typing on a large collection of isolates for the first time and demonstrated a larger genetic variety compared to methods as *spa* typing and PFGE (approximately one genotype per farm). This variety raised the question whether this method is relevant and useable for molecular epidemiology of MRSA isolates of pigs.

First, the genetic basis of the MLVA method is the length of five repeat regions in the coding sequences. It remains to be elucidated whether these repeat regions are the most approriate ones to use. It has been reported that some repeat regions such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) adapt really fast, since they play a role in the "immunity" of the prokaryotic cell against bacteriophages (Bhaya et al., 2011; Westra et al., 2012). When the studied loci evolve too fast, the method becomes too discriminatory and the epidemiological value uncertain. As a control, we investigated whether the MLVA type of eight isolates changed *in vitro* over a ten-day period with repeated subculturing at 37°C and observed no differences throughout time (data not shown). This means that the MLVA type is stable under laboratory conditions. However, this does not rule out the possibility of fast evolution *in vivo* under farm conditions. To asses this, it might be interesting to perform colonization experiments to see how fast new MLVA types are generated in animals. Preferably antimicrobial treatments, movements and mingling of the pigs should be included in these studies to imitate farm conditions.

Second, the interpretation method of the results plays an important role. Changing the settings of the program, using a cut-off value and clustering of the MLVA types were the possibilities that have been considered and analyzed. In the end, after generating a dendrogram using UPGMA, we clustered the closely related MLVA types (i.e. MLVA types differing in one repeat region). This resulted in less variety and more interpretable results. Doing so increased the value of MLVA typing to a higher extent than PFGE as seen in chapter IVb (within one pulsotype various MLVA types were located). So, our MLVA typing scheme with an appropriate cluster analysis is a good method to study the epidemiology of LA-MRSA.

Thorough molecular typing on a large collection of isolates allowed us to assess whether it is necessary to use all different methods or not. Seen the cost, time and specific equipment of some methods, this is an important question. When studying transmission within one entity, methods such as *spa* typing, PFGE or MLVA typing are often used. During *spa* typing, only one region of the genome is sequenced, whereas more regions are analyzed in PFGE (various cutting sites of the enzyme) and MLVA (at least five repeat regions). In case of PFGE, no

information is available on what changes occurred in the genome. It was obvious that *spa* typing was far less discriminatory as the other two techniques (Diversity index of 0.37 for *spa* typing, 0.87 for PFGE with *BstZ*I and 0.98 for MLVA). Compared to PFGE, a larger genetic diversity was observed after MLVA typing throughout the various chapters. When studying the genetic diversity of a large collection of isolates (as in chapter IVb), the high throughput and fast methodology of MLVA typing is an important advantage of this method. Using MLVA typing allowed us to gain insights in the genetic diversity of the isolates within one entity. In such way, MLVA typing is certainly a good method for studying the genetic diversity of isolates. In addition, this is a high throughput PCR-based and low time-consuming method compared to PFGE, which makes it an interesting method for future work. As mentioned in section 3.4., a combination of methods is often used for studying the molecular epidemiology. In my opinion, combining PFGE and MLVA typing would be a good option seen both the individual discriminatory powers observed in the present work, the combination of a PCR and non-PCR based method and the obtained results (in some MLVA clusters, more than one pulsotype was identified).

LA-MRSA was found throughout the pork production chain. Is it possible to reduce the amount of LA-MRSA in the pork production chain by means of hygienic measures?

At present, no standard protocol for hygienic measures is available for the reduction of LA-MRSA at **farm level**, as was evident from our survey on the four longitudinally sampled farms. Although farmers C and D applied cleaning and disinfection strategies, high MRSA prevalences were still observed. In addition, a preliminary study indicated that sow washing, as performed on the farm, did not seem to influence the sows' skin MRSA status (Verhegghe et al., 2013c). Merialdi et al. (2012) and Pletinckx et al. (2013b) reported that cleaning and disinfection was able to reduce the number of MRSA-positive environmental samples, but could not eliminate MRSA completely from the animals. When considering all available data on LA-MRSA, it can be concluded that it will be difficult to eradicate this bacterium completely from a farm once it is present. Nevertheless, it might be interesting to consider creating one standard protocol for cleaning and disinfection that reduces not only the LA-MRSA load on a farm but also the load of other pathogens such as *Salmonella*. Further research could start with *in vitro* experiments to determine for example: the bactericidic concentrations of disinfectants; whether the bacterium is able to become resistant to these concentrations and the influence of various construction materials on the survival of bacteria. Subsequently, experimental stable settings could be used to confirm the results. In addition, a

rotating disinfection scheme should be considered to reduce the possibility of bacteria acquiring resistance. Last, the proposed schemes should be tested in the field on pig farms. This scheme should also be easy-to-use and cost-effective. Another more cost-effective option is to decrease the MRSA load on pigs leaving the farm. When finishing pigs are disinfected upon transport to the slaughterhouse, the initial MRSA-source is reduced and this may result in lower MRSA numbers on carcasses and meat.

Another option might be competitive exclusion. Hereby, pigs are inoculated with other harmless bacteria, competing for the same niche on the animal. An interesting observation and difference between the low and high colonization farms was the presence of *Staphylococcus sciuri* in the nares of the piglets in the nursing units of farms A and B, which is a known colonizer of pigs (data not shown). It might be interesting to investigate whether *S. sciuri* is indeed able to inhibit MRSA colonization. However, care should be taken with antimicrobial use since we have noticed that upon moving to another unit and treatment with antibiotics, these susceptible bacteria disappeared and the percentage of MRSA colonized animals increased, which might be related.

Before the hygienic measures in the slaughterhouse can be considered, the question remains where the detected MRSA on the pig carcasses is originating from.

Finding MRSA on the carcasses after cooling is an indication of survival of MRSA throughout the slaughterhouse or slaughter process. There are some critical points in the slaughter process. After bleeding out of the carcass, scalding (+/- 4 minutes) occurs during which carcasses sink into water (60°C) up till the throat. The remaining part of the carcass is sprayed with the same water (60°C). The temperature should be high enough to reduce *S. aureus/*MRSA as upon milk pasteurization (1 minute-60°C) a 2 log reduction of *S. aureus* N12 was observed (Pearce et al., 2012). However, Kennedy and coworkers (2005) reported that some *S. aureus* strains have a decimal reduction time of 5 to 6 minutes at 60°C, which implies that *S. aureus*/MRSA could survive scalding. In addition, warm water results in formation of aerosols in which MRSA is able to survive, resulting in recolonization and cross contamination of the carcasses (Liu et al., 2012). After scalding, the carcasses are tumbled and sprayed with water (+/- 40°C) for dehairing. Here, surviving MRSA could spread over the carcass and the equipment, forming a MRSA source for following carcasses. After dehairing, the carcasses enter another area where brushing and singeing of the carcasses happens. Singeing is known to reduce total plate counts with 4 to 5 log (Bolton et al., 2002; Pearce et

al., 2004; Spescha et al., 2006). However, when observing this process and the carcasses afterwards, it appears that singeing of the forelimbs does not occur efficiently and occasionally, a carcass is not singed. Subsequently, the carcasses are brushed again with water. Once MRSA is present in this equipment, cross-contamination on other carcasses could happen. Afterwards the carcasses enter the “clean area” of the slaughterhouse where evisceration of the carcasses occurs. Since MRSA is rarely found in the intestines of pigs, this will not likely cause contamination of the carcasses. In the clean area, the lower part of the carcasses sometimes touches the walls, which may induce environmental contamination during the slaughter day. In addition, the atmosphere of the slaughterhouse is quite humid, which could induce the environmental spread of MRSA, which has already been reported in pig slaughterhouses (Van Cleef et al., 2010b; Gilbert et al., 2012). Upon manipulation of the carcasses, slaughterhouse workers could also cause cross-contamination between carcasses. However, knives and equipment are sterilized frequently (every five carcasses). Afterwards, the carcasses are cooled for approximately 2 hours. After cooling, the carcasses are manually pushed in the cooling rooms, which again makes human contamination of carcasses possible. At present, few data is available on these critical points in the process. To gain more insights into the MRSA spread in the slaughterhouses, more sampling events are needed of carcasses throughout the process, of carcasses originating from different herds, on various time points throughout the slaughter day and of the slaughterhouse personnel. The obtained MRSA isolates should be typed thoroughly to demonstrate the possible cross-contamination.

One has to be certain that the hygiene procedure of the slaughterhouse is capable of eliminating/reducing all MRSA present since we found MRSA on the carcasses at the beginning of the slaughter day.

At present, the focus of the hygiene management of slaughterhouses is on fecal excreted organisms such as *Salmonella* and Shiga-toxin producing *Escherichia coli*, which are harmful for humans. It should be confirmed whether the management practices for these intestinal bacteria are also useable for non-fecal excreted bacteria, such as MRSA. At the studied slaughterhouse, various points of the slaughter line are being tested for total plate count and the presence of *Enterobacteriaceae* after cleaning and disinfection (personal communication with hygiene manager of the slaughterhouse). It could be interesting to add *S. aureus* as an indicator organism for non-fecal excreted and environmental organisms. Most disinfection products are more efficient against *S. aureus* than most gram-negatives. When the general hygiene procedure is able to decrease the numbers of gram-negatives, it is most likely that

S. aureus/MRSA is also decreased. Caution is still needed as some MRSA ST9 strains carried disinfection resistance genes (Wong et al., 2013). Alternate use of different disinfection products could also be interesting to prevent bacteria of getting resistant. A major problem of cleaning and disinfection of the equipment is the complex structure of some devices. For example, it takes two hours to clean and disinfect the dehairing device at the studied slaughterhouse (personal communication with hygiene manager of the slaughterhouse). Due to the complex nature of devices, it is possible that bacteria survive in the equipment.

Survival of MRSA on carcasses will eventually result in the presence of MRSA in **butcheries** and on **pork**. When MRSA subsequently persists in the butchery, this bacterium might contaminate other meat products and the butcher, who may be a vector for contaminating other meat products. Our results show that there is an import of various MRSA strains in a butchery, since diverse MRSA genotypes were isolated from pork, originating from the same butchery (Chapter Vb). Nevertheless, a lot of information is missing on the presence of MRSA in butcheries and the potential transmission of this bacterium throughout the butchery. Transport of carcasses from the slaughterhouse or meat processing plant to the butchery should also be investigated. According to the sectorial guide G-003 (2005), cleaning and disinfection of the workplace and store should be performed on daily basis. Similar to the slaughterhouses, it should be investigated whether the proposed hygienic measures are able to reduce the bacterial load of MRSA in the butchery or not.

Can LA-MRSA be transmitted from the pork production chain to humans and which protective measures can be taken?

It is known that farmers, slaughterhouse workers and to a lesser extent farm veterinarians have an increased risk of LA-MRSA carriage as they come in contact with living pigs (Graveland et al., 2010; Van Cleef et al., 2010b; Garcia-Graells et al., 2012). **Farmers** spend a lot of time in the barns and come in close contact with the pigs, the air and dust. The use of personal protection devices (PPD) such as aprons, gloves and a mask or hand disinfection on a regular basis should be considered. However, it was reported that the use of these protective measures increases the risk for MRSA carriage (Wulf et al., 2007; Denis et al., 2009). Wulf and co-workers (2007) suggested that this was due to inconsistent use of these measures (not using the measures at all times or reusing for example dust masks) or impropriate (hand) hygiene afterwards. Moreover, recolonization through the outgoing air was also suggested. Consistent and correct use of PPD is recommended since fast recolonization of the farmers

will occur upon contact with the animals (Köck et al., 2012). Lowering the MRSA load on the farm will possibly result in lower colonization rates of the farmer.

Slaughterhouse workers wear aprons and hair nets and upon entry of the slaughter area, hands and boots are disinfected. These measures are applied to prevent the workers to contaminate the carcasses, rather than protecting them from the bacteria present on the carcasses. **Butchers** also wear aprons for general hygiene in the butchery in addition to good hand and personal hygiene. Van Cleef and coworkers (2010b) found LA-MRSA on slaughterhouse workers in contact with living animals and not in persons working in the meat processing area. From 300 Chinese butchers (nasal samples), Boost and coworkers (2012) found 17 MRSA strains of which ten CC9. When considering the few existing data, it can be assumed that meat handlers and butchers are at lower risk of getting contaminated with LA-MRSA. Still, the colonization status of these persons should be studied to determine the actual risk of contracting MRSA from carcasses or meat and to confirm whether this professional group represents an additional risk group for LA-MRSA carriage or not. Still, a good protection measure is a good hand and personal hygiene in addition to good hygienic measures to clean the butchery.

The risk of MRSA ST398 transmission from a risk group to the **general human population** is expected to be low, since it was observed that MRSA ST398 is 2.9 times less transmissible than non-ST398 MRSA (Bootsma et al., 2011). Caution is needed when coming in close contact with a farm. High isolation rates were observed in the outgoing air of the stables. A private farm visit might therefore result in contact with LA-MRSA, but in humans with sporadic pig contact, decolonization occurs within 24 hours (Van Cleef et al., 2011; Bissdorff et al., 2012; Frana et al., 2013). Pork can be considered as potential source of LA-MRSA for the general human population, since LA-MRSA was isolated by direct plating from 8% of the samples and after enrichment from 72% of the samples. After manipulating meat, good hand hygiene is needed. In addition, cleaning of the kitchen tools should be kept in mind to prevent transmission to other food products and throughout the household. Even though MRSA is killed after heating of the meat, more information is needed on the potential of LA-MRSA to spread throughout the kitchen and the household (Nunan and Young, 2007; Haenen et al., 2009; Davies et al., 2011; Lekkerkek et al., 2011; Wassenberg et al., 2011). As only few MRSA ST398 strains have been described with enterotoxin genes, foodborne illness will not likely occur (Schijffelen et al., 2010).

Should LA-MRSA transmission from the pork production chain to the general human population be considered as a potential public health hazard now and in the future?

At present, some reports describe the presence of MRSA ST398 infections in humans with and without contact with animals. As mentioned in sections 2.6.7.4. and 2.6.8 (chapter 1), these infections vary from mild to severe. Seen the current low virulence and low transmission possibility of MRSA ST398, at present, this MRSA type might be considered as a minor threat for human health. A recent Belgian study estimated the prevalence of MRSA carriage upon hospital admissions from 2006 to 2008 and demonstrated that only 2% of all isolated MRSA strains were LA-MRSA (Vandendriessche et al., 2012). Caution is still needed.

Thorough analysis of various genomes of LA-MRSA strains indicated that this bacterium has a rather flexible genome, which means that this bacterium is very receptive for new genes (Schijffelen et al., 2010: Price et al., 2012; Golding et al., 2012). Some examples of this feature are given below. First, LA-MRSA has been associated with multiple SCC*mec* types, which is in contrast to HA- and CA-MRSA where this variability is much lower (see chapter 1-section 2). Second, we found a great variety in MLVA types (see above). Third, LA-MRSA is able to take up and carry various antimicrobial resistance genes, other than genes associated with a SCC*mec* cassette, originating from its environment (Katayama et al., 2003; Tulinski et al., 2011). A fourth important example of the flexibility of the LA-MRSA genome is demonstrated by the variable presence of virulence genes. Baquero (2012) described that upon adaptation to pigs, LA-MRSA lost genes associated with human virulence. At present, some LA-MRSA strains have been isolated with genes associated with human, bovine or avian virulence (Schijffelen et al., 2010; Monecke et al., 2013; Petersen et al., 2013). This fact emphasizes the potential danger for the human population. Other genes which form a potential threat for humans are PVL (a known virulence factor in CA-MRSA strains) and enterotoxin genes (often causing foodborne diseases upon uptake of the toxins). Description of LA-MRSA strains carrying the latter two is again worrisome for the general human population (Jamrozy et al., 2012; Osadebe et al., 2012).

These findings indicate that LA-MRSA and MRSA ST398 in particular may have the potential to become more virulent and more pathogenic for humans in the future. As mentioned before, pigs represent a large LA-MRSA reservoir and these changes may have a large impact on public health. Finding one predominant MRSA ST398 clone throughout the

pork production chain might indicate that this clone is better adapted to the chain than others. When this clone comes in contact with other bacteria (for example a risk group person entering the hospital), it is not unlikely that uptake of certain genes (antimicrobial resistance, virulence or enterotoxin genes) happens, resulting in a more virulent or more resistant clone. When this clone subsequently comes in contact with the pigs again, a more virulent or resistant LA-MRSA could spread fast as demonstrated in transmission studies. This underlines the need of reducing the LA-MRSA load on farms and subsequently in slaughterhouses (see above). In addition, it highlights the need of screening persons belonging to LA-MRSA risk groups upon admission in the hospital as already done in for example The Netherlands. Moreover, farmers should be aware of the fact that they are potential carriers of LA-MRSA, especially upon hospital admission or when suffering from chronic diseases.

In the future, the presence of LA-MRSA in hospitals should be followed and the genomes should be studied to track possible changes in virulence or antimicrobial resistance in these strains. A change in antimicrobial resistance was already observed during our pork study: 24 MRSA CC398 isolates that were sensitive to tetracycline, despite the carriage of the tetracyclin resistance genes. In addition, the presence of MSSA ST398 in for example livestock, rodents and the human population should be more thoroughly investigated as this MSSA also poses a potential source for MRSA ST398 upon acquisition of a SCC*mec* cassette.

Conclusions

From this PhD thesis, it can be concluded that MLVA is a good method for studying the molecular epidemiology of MRSA ST398. Using various typing methods allowed us to identify one dominant MRSA clone which is apparently widespread throughout the pork production chain.

The MRSA prevalence was remarkably higher in pigs than in poultry and cattle. Moreover, longitudinal samplings revealed various trends in MRSA carriage of the pigs. The MRSA status of the sow at farrowing played an important role in the MRSA status of her offspring. We also identified mother sows, the environment and other pig(let)s as important MRSA sources for pig(let)s. MRSA ST398 was isolated from different pork types and pig carcasses, of which the forelimb appeared to be a good sampling location.

In conclusion, we can state that MRSA ST398 is widespread throughout the pork production chain and it represents a large source for the general human population. At present, the transmission risk to this population appears low, but seen the proven capacity of rapid genetic changes in the pig niche and the potential of this bacterium to acquire virulence and antimicrobial resistance genes, caution is needed for the future.

Summary

Few years after the first clinical use of the antibiotic methicillin, methicillin-resistant *Staphylococcus aureus* (MRSA) strains were reported. At present, three MRSA types have been described: the hospital-associated MRSA (HA-MRSA), the community-associated MRSA (CA-MRSA) and more recently the livestock-associated MRSA (LA-MRSA). LA-MRSA, which consists mainly of MRSA Sequence Type (ST) 398 in Europe has been isolated from livestock animals, especially pigs and to a lesser extent cattle. MRSA ST398 is considered as a minor animal health problem since only few MRSA ST398 infections have been described in pigs. The description of MRSA ST398 colonization and sporadic infections in humans, especially those who have been in contact with pigs, indicated that pigs might be a potential MRSA source for the general human population. At present, information is lacking on possible transmission routes of MRSA ST398 to the general human population. Moreover, insights into potential MRSA ST398 sources and transmission routes may help to create and implement control measures to reduce the amount of MRSA ST398 along the pork production chain.

In **Chapter I**, a literature review is given on the evolution of *S. aureus* to MRSA and the three MRSA types (HA-MRSA, CA-MRSA and LA-MRSA) and their main characteristics are discussed. A description of possible transmission routes of (LA-) MRSA from a pig farm to the human population is given. MRSA ST398 isolation and confirmation are assessed. Subsequently, an overview of molecular typing methods, their (dis)advantages and their use in the molecular epidemiology of MRSA ST398 is given. Last, the Belgian pork production chain is discussed.

The general and specific aims are described in **Chapter II**. The general aim was to gain insights into the molecular epidemiology of MRSA ST398 throughout the Belgian pig production chain. More specific, pig and multispecies farms were screened to detect differences between the MRSA carriership of pigs, poultry and cattle. Farrow-to-finish farms were sampled longitudinally to determine the effect of sows and environment on the piglets' MRSA status. The MRSA presence on pig carcasses and pork was assessed. On all obtained isolates, a molecular typing was performed to gain insight into the genetic diversity.

The first study (**Chapter III**) involved the screening of 30 Belgian farms (10 pig, 10 pig/poultry and 10 pig/cattle farms) for the presence of LA-MRSA. The aims of the study were i) to gain insight into the distribution of LA-MRSA in pig farms versus multispecies farms, ii) to examine the genetic diversity of the obtained isolates in these farm types, iii) to

determine whether poultry and cattle carry other STs than ST398 and iv) to asses any correlation between pigs and other species reared on one farm. On each farm, 10 nasal swabs were taken from pigs. When present, cattle (n=10) were sampled in the nares and poultry (n=10) in the nares, earlobes and cloaca. MRSA was isolated from pigs on 26 out of 30 farms. On one pig/poultry and five pig/cattle, MRSA was detected in poultry and cattle, respectively. Molecular typing of the obtained isolates (n=170) revealed the presence of eight *spa* types (t011, t034, t567, t571, t1451, t2974, t3423 and t5943) and SCC*mec* cassette types IVa and V were present in 20% and 72% of the isolates, respectively. Combining the results of Pulsed Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable-number tandem-repeat Analysis (MLVA) revealed the presence of 18 genotypes of which one genotype predominated (56% of the positive farms). All isolates were resistant to tetracycline and resistance to other antimicrobial agents such as trimethoprim was frequently observed. No significant effect between the farm types and no correlation between the number of positive cows or poultry and the number of positive pigs was observed. In addition, only MRSA ST398 was found with a large genetic diversity within certain farms when combining different typing methods. Poultry and cattle did not carry other STs than ST398.

A longitudinal study (**Chapter IV**) was conducted on four farrow-to-finish farms (farms A to D), selected from the thirty screened farms (**Chapter III**). The aims of this longitudinal study were i) to determine the age at which piglets become colonized with LA-MRSA, (ii) to assess the possible effect of the sow colonization status on their offspring colonization status and (iii) to examine the effect of the environment (**Chapter IVa**). Moreover, molecular typing occurred on a selection of the obtained isolates iv) to gain insight into the strain carriage of animals throughout time and v) to determine potential MRSA sources for the pigs (**Chapter IVb**). On each farm, nasal swabs from 12 sows and their offspring were collected during a 6-month period. Sows and their offspring were sampled throughout the nursery period. The piglets were additionally sampled after weaning, before and after moving to the finishing unit and at slaughter age. At every sampling event, the environment of one pen (wall, floor and air) was also sampled. Two MRSA colonization profiles were observed. On the low colonization farms (A and B), the sow colonization prevalence remained low in the nursing unit (max. 17% and 33% of the sows, respectively). Moreover, the piglet colonization prevalence remained low in the nursing unit (farm A: 0-7% of the piglets, farm B: 0-36%) and increased at the end of the piglets' stay in the growing unit (farm A: 91%, farm B: 69%). On the high colonization farms (C and D), the sows' and piglets' colonization prevelances were

high from the beginning of the sampling events and reached 100% before weaning. A decrease in colonization to a prevalence of approximately 50% on farm A and 80% on farms B to D was observed towards slaughter age. The colonization age differed amongst the farms from 0.1 days and 24 days on farms C and B, respectively, to 46 days on farm A. A statistically significant effect of the sow status at farrowing on the piglets' status was observed. When high or low MRSA prevalences were observed in piglets, high or low MRSA detection occurred in their environment and *vice versa*. On a selection of obtained sow, piglet and wall isolates (n=964/3450), MLVA typing was performed and afterwards PFGE, *spa* typing and SCC*mec* typing. In contrast to one *spa* type, one SCC*mec* type and one or few pulsotype(s), a large variety of MLVA types was observed on the four farms, clustered together in one cluster on farms A and B, four on farm C and two on farm D. These clusters were detected in piglets at various sampling occasions. In comparison to their mother sow, piglets carried both related and unrelated MLVA types at farrowing and onwards. In the environment, a mixture of sow, piglet and environmental MLVA types was observed. This longitudinal study indicated that the sow's colonization status is of importance for the piglet colonization status. Besides the mother sow, other factors such as the environment and the piglets themselves can be considered as a potential MRSA source for piglets. The finding of few dominant MLVA types with a lot of related types within one farm indicates that a farrow-to-finish farm can be considered as a closed system in which one or few MRSA strain(s) evolve.

Next, the presence of MRSA on pig carcasses in the slaughterhouse (**Chapter Va**) and pork (**Chapter Vb**) was assessed. Carcasses, originating from farm B (**Chapter Va**), were sampled in the slaughterhouse after cooling i) to determine the best sampling location for MRSA (ST398) on a carcass. PFGE was used ii) to study the genetic variability of the obtained isolates and to compare the diversity with the herd pulsotype. Forty carcasses were sampled at six locations: ham, abdomen, back, forelimb, sternum and intestine cavity. On each site, 100 cm² was sampled using a sponge. Before enrichment, no MRSA was isolated from the carcasses whereas after enrichment, MRSA ST398 was isolated from 19 carcasses and in 16 cases the forelimb was MRSA-positive. PFGE revealed the presence of three pulsotypes of which the dominant type was also the herd pulsotype. During a second experimental study, retail pork samples (chops, bacon, minced pork, ribs, forelimbs and ears) were collected from four butcheries (A to D) during 6 successive weeks (n=137) to iii) determine the MRSA prevalence on Belgian pork and iv) to assess whether butcheries are a potential MRSA source

or not (**Chapter Vb**). Twenty-five grams of chops, bacon and minced meat were 10-times diluted. The ribs, forelimbs and ears underwent a 1/1 dilution and the cm^2 per sample was determined. Each sample was homogenized and a 10-fold dilution series was made (after removal of the ribs, forelimb and ears). Each sample was spread plated on a chromogenic MRSA selective medium both before and after enrichment. Direct plating of the dilution series resulted in a MRSA prevalence of 8% with colony counts ranging from 6 to 80,000 cfu/g or cfu/cm^2. After enrichment, 72% of the samples were MRSA-positive and highest isolation rates were seen on ear, forelimb and rib samples. Molecular typing revealed that in each butchery the MRSA isolates were highly diverse. Large differences were seen when comparing the MRSA prevalence before and after enrichment of the carcass and pork samples. The recovery of the herd pulsotype on the carcasses indicates that transmission from the living pigs to the carcasses occurs along the slaughterline. In addition, a butchery can be considered as a reservoir in which genetic diverse isolates are present and persist.

In **Chapter VI**, the results obtained in the previously described experiments are critically evaluated and future perspectives are formulated.

In general, during this doctoral research data were collected on the diversity of MRSA ST398 along the pork production chain, from nursery to butchery. In summary, besides the mother sow, other MRSA sources for piglet contamination such as the environment and other piglets are present within a farm. Moreover, within one farm, few dominant genotypes were isolated, which appeared to evolve within their "niche". MRSA ST398 was also isolated from pig carcasses and pork. Improvement of existing or creation of new control measures are needed to reduce the amount of MRSA ST398 on Belgian pig farms and subsequently to reduce the transmission risk of MRSA ST398 to the human population.

Samenvatting

Kort na het eerste klinische gebruik van het antibioticum methicilline werden de eerste methicilline-resistente *Staphylococcus aureus* (MRSA) stammen beschreven. Tot op heden bestaan er drie MRSA types: ziekenhuisgebonden MRSA (HA-MRSA), gemeenschapsgebonden MRSA (CA-MRSA) en diergebonden MRSA (LA-MRSA). Deze laatste, waartoe MRSA Sequentie type (ST) 398 behoort, wordt vooral bij landbouwdieren (hoofdzakelijk bij varkens en vleeskalveren) gedetecteerd. Varkens worden vaak als dragers van MRSA ST398 beschouwd, daar zij zelden MRSA infecties vertonen. De beschrijving van MRSA ST398 kolonisatie en sporadische infecties bij mensen die al dan niet in contact kwamen met varkens, doet vermoeden dat varkens een potentiële bron zijn van overdracht van MRSA ST398 naar de mens. Kennis omtrent mogelijke MRSA bronnen, eventuele contaminatieroutes en uiteindelijke transmissie naar de gemeenschap, is vandaag evenwel beperkt aanwezig. Deze kennis zou toelaten om bepaalde kritische punten in de varkensvleesproductieketen te identificeren en zo de insleep van MRSA in de gemeenschap te beperken of te voorkomen via remediëringsmaatregelen.

In **hoofdstuk I** wordt een overzicht van de literatuur geboden betreffende de evolutie van *S. aureus* naar MRSA. Hierbij worden de drie MRSA types en hun eigenschappen besproken. Daarnaast wordt dieper ingegaan op de mogelijke (LA-)MRSA transmissieroutes tussen de varkensvleesproductieketen en de gemeenschap. Het volgende deel bestaat uit een overzicht over de isolatie, identificatie en typering van MRSA ST398. Hierbij worden de gebruikte moleculaire technieken besproken en de toepassing ervan in de moleculaire epidemiologie wordt toegelicht. Tenslotte wordt een overzicht gegeven van de Belgische varkensvleesproductieketen.

In **hoofdstuk II** worden de doelstellingen van dit werk toegelicht. Het hoofddoel van dit doctoraat was het verkrijgen van meer inzichten in de verspreiding van LA-MRSA doorheen de varkenshouderij door het uitvoeren van een moleculaire epidemiologische studie. Specifiek werd gekeken naar verschillen in MRSA dragerschap van varkens, kippen en runderen door een screening uit te voeren van varkens- en gemengde bedrijven. Een longitudinale studie werd opgezet om een mogelijke invloed van de MRSA status van de zeugen en/of de omgeving na te gaan op de MRSA status van een big. Daarenboven werd de prevalentie van MRSA op varkenskarkassen en varkensvlees bepaald. Er werd een moleculaire typering uitgevoerd op alle isolaten om zo de nodige inzichten te verwerven in de genetische diversiteit ervan en de potentiële MRSA bronnen.

Tijdens een eerste studie (**hoofdstuk III**) werden 30 Belgische bedrijven (10 varkens-, 10 varkens-pluimvee en 10 varkens-rundvee bedrijven) gescreend met als doel i) de aanwezigheid van diergebonden MRSA op de drie bedrijfstypes te vergelijken, ii) de genetische diversiteit te bepalen binnen één bedrijf en te vergelijken tussen de bedrijven, iii) na te gaan of pluimvee en runderen andere ST dragen dan ST398 en iv) een mogelijke correlatie na te gaan tussen MRSA dragerschap van varkens en de andere diersoorten aanwezig op het bedrijf. Op elk bedrijf werden stalen genomen van 10 dieren van elke aanwezige diersoort. Bij varkens en runderen werd een neusswab afgenomen en bij kippen een staal van de neusschelp, oorlel en cloaca. In totaal werd op 26 van de 30 bedrijven MRSA geïsoleerd bij de varkens, op 5 varkens-rundvee bedrijven bij runderen en op 1 varkens-pluimvee bedrijf bij kippen. De 170 verkregen MRSA ST398 isolaten behoorden tot 8 *spa* types (t011, t034, t567, t571, t1451, t2974, t3423 and t5943). SCC*mec* cassettes IVa en V werden respectievelijk bij 20% en 72% van de isolaten teruggevonden. Combinatie van Pulsed Fiel Gel electrophorese (PFGE) en Multiple-Locus Variable-number tandem-repeat Analyse (MLVA) toonde aan dat één van de achttien genotypes dominant was (56% van de positieve bedrijven). Alle isolaten vertoonden verworven resistentie tegenover tetracycline. Daarnaast werden ook hoge resistentie precentages vastgesteld ten opzichte van andere antimicroniële middelen zoals trimethoprim. Tijdens deze studie werd er geen statistisch significant effect gezien van het bedrijfstype op de MRSA status van de varkens. Er was wel een statistisch significant verschil tussen de MRSA isolatie bij varkens en de isolatie bij rundvee/pluimvee aanwezig op hetzelfde bedrijf. De combinatie van verschillende typeringsmethodes toonde een enorme variatie aan in de MRSA ST398 isolaten, aanwezig op het bedrijf. Kippen en runderen droegen geen andere STs dan ST398.

Na screening van deze 30 bedrijven (**hoofdstuk III**) werden 4 bedrijven geselecteerd voor een diepgaande longitudinale studie met als doel i) de initiële kolonisatieleeftijd van biggen te bepalen, ii) het effect van de MRSA status van de zeug op haar biggen na te gaan en iii) het effect van de omgeving te bepalen (**hoofdstuk IVa**). Daarnaast werd er een selectie van isolaten verder getypeerd om iv) het dragerschap van de biggen doorheen de tijd en v) mogelijke MRSA bronnen te bepalen (**hoofdstuk IVb**). In de kraamstal van elk bedrijf werden neusswabs genomen van 12 zeugen en hun biggen vanaf de geboorte tot het spenen. Bijkomend werden de biggen bemonsterd na het spenen, voor en na het verplaatsen naar de afmestafdeling en op slachtleeftijd. Bij elke staalname werd ook de omgeving van één hok bemonsterd (wand, vloer en lucht). Twee kolonisatieprofielen konden onderscheiden worden.

Op bedrijven A en B, gedefinieerd als “lage kolonisatie” bedrijven, werd een lage prevalentie gevonden bij de zeugen (maximum 17% en 33% van de zeugen op bedrijven A en B). Bij de biggen bleef de prevalentie laag (A: 0-7% van de biggen en B: 0-36%). Pas op het einde van de biggenbatterij periode werd bij respectievelijk 91% en 69% van de biggen op bedrijven A en B MRSA geïsoleerd. Op de “hoge kolonisatie” bedrijven C en D waren de isolatiepercentages hoog vanaf het werpen en werd voor spenen zelfs bij 100% van de dieren MRSA geïsoleerd. Op alle bedrijven zakte de prevalentie naarmate de slachtleeftijd naderde: tot 50% op bedrijf A en rond de 80% bij bedrijven B, C en D. De kolonisatieleeftijd verschilde van bedrijf tot bedrijf gaande van 0,3 dagen op bedrijf C tot 46,6 dagen op bedrijf A. Er werd een statistisch significant effect gevonden van de zeugstatus bij werpen op de big status. Bij een hoge/lage MRSA prevalentie bij de biggen, werd er veel/weinig MRSA in de omgeving gevonden en omgekeerd. De molecularie typering op een uitgebreide selectie van isolaten wees uit dat er slechts een paar dominante types aanwezig waren binnen één bedrijf. Die types werden door de meeste biggen doorheen de tijd gedragen. Biggen droegen zowel types die verwant waren als types die niet verwant waren aan aan de moederzeug. Deze resultaten wijzen op het belang van de zeug, de omgeving en de biggen zelf als mogelijke MRSA bronnen voor biggen. Een varkensbedrijf is mogelijks een gesloten systeem waarbinnen één of enkele dominante MRSA types zich handhaven.

In **hoofdstuk V** werden staalnames uitgevoerd op varkenskarkassen (**hoofdstuk Va**) en op varkensvlees (**hoofdstuk Vb**). In het slachthuis werden de karkassen van één groep vleesvarkens, afkomstig van bedrijf B (**hoofdstuk Va**) bemonsterd met als doel i) de beste staalnameplaats voor MRSA ST398 te bepalen en ii) de genetische diversiteit van de verkregen isolaten te bestuderen. Van 40 karkassen werden op drie plaatsen stalen genomen aan de buitenkant (achterbeen/hesp, buik en rug) en op drie plaatsen aan de binnenkant (darmholte, sternum en voorpoot). Honderd cm^2 per staalnameplaats werd geswabd met behulp van een sponsje. Na rechtstreekse incubatie van de sponsjes werd geen MRSA gedetecteerd. Aanrijking van de sponsjes daarentegen zorgde voor MRSA isolatie van 19 karkashelften waarvan bij 16 karkassen op de voorpoot. Er werden drie pulsotypes gevonden, waarvan het dominante type het varkensbedrijftype was. Tijdens een tweede studie werd er gedurende 6 weken wekelijks bij twee slagers en twee supermarkten versneden varkensvlees (n=137) gehaald, zijnde mignonette, spek, varkensgehakt, ribbetjes, poten en oren (**hoofdstuk Vb**). Dit had als doel iii) de MRSA prevalentie op Belgisch varkensvlees te achterhalen en iv) na te gaan of slagerijen een bron zijn voor de contaminatie van varkensvlees met MRSA.

Mignonettes, spek, varkensgehakt (25g) werden 1/10 verdund. De ribbetjes, poten en oren werden 1/1 verdund en de oppervlakte van het vleesstaal werd bepaald. Na homogenisatie (en verwijderen van de ribbetje, poten en oren) werd er een 10-voudige verdunningsreeks gemaakt. Alle verdunningen werden uitgeplaat voor en na aanrijking. Zonder aanrijking waren 8% van de stalen MRSA positief met 6 tot 80000 kolonievormende eenheden per gram (voor mignonette, spek en gehakt) of per cm^2 (voor ribbetje, poten en oren). Na aanrijking werd er op 72% van de stalen MRSA gevonden vooral op de oren, poten en ribbetjes. Een grote genetische variatie werd gezien in isolaten binnen elke slagerij. Het terugvinden van het varkensbedrijftype op de karkassen wijst aan dat er besmetting van de karkassen gebeurt doorheen de slachtlijn. Een slagerij kan beschouwd worden als een reservoir voor verschillende genetisch diverse isolaten.

In **hoofdstuk VI** werden de resultaten van de experimentele studies kritisch geëvalueerd en werden toekomstperspectieven geformuleerd.

Samengevat biedt het werk in dit proefschrift meer inzichten in de genetische diversiteit van MRSA ST398 binnen de varkenshouderij, gaande van kraamstal tot slagerij. Algemeen werd gesteld dat naast de zeugen, ook andere MRSA bronnen, zoals de omgeving en andere biggen, een rol kunnen spelen in de contaminatie van biggen. Daarnaast bleek dat binnen één bedrijf enkele dominante stammen aanwezig zijn die binnen hun niche evolueren. Tevens werd er MRSA geïsoleerd van varkenskarkassen en varkensvlees. Het creëren van efficiënte hygiënemaatregelen is nodig om MRSA ST398 te reduceren in Belgische varkensbedrijven, zodat de transmissie naar de humane populatie via de varkenshouderij beperkt blijft.

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Curriculum Vitae

Marijke Verhegghe werd geboren op 1 november 1980 te Etterbeek. Na het behalen van de graad wiskunde-wetenschappen aan het Sint-Niklaas Instituut Anderlecht in 1998, vatte ze het graduaat in de medische laboratorium technologie aan op de Erasmushogeschool Brussel, waarin ze in het academiejaar 2000-2001 met grote onderscheiding haar diploma haalde. Hierna vatte ze de 2^{e} kandidatuur aan aan de Vrije Universiteit Brussel. Na het behalen van de graad van kandidaat in de Biologie in het academiejaar 2001-2002, vatte ze de licenties Biologie optie Cel-en Ontwikkelingsbiologie aan, waarin ze in het academiejaar 2003-2004 met grote onderscheiding haar diploma haalde.

Tot december 2008 werkte ze als medisch laborant bij Medic Lab te Aalst. In januari 2009 startte ze met een doctoraatsonderzoek aan het Instituut voor Landbouw en Visserij Onderzoek (ILVO), Eenheid Technologie en Voeding. In samenwerking met de vakgroep Pathologie, Bacteriologie en Pluimveeziektenvan van de Universiteit Gent, het Katholieke Hogeschool Zuid-West Vlaanderen Associatie KULeuven (KATHO) te Roeselare en het Centrum voor Onderzoek in Diergeneeskunde en Agrochemie (CODA) te Ukkel werkte zij aan een IWT project getiteld “Studie van contaminatiepatronen en kiem-gastheer interacties ter beheersing van MRSA bij varkens en andere nutsdieren”.

Marijke Verhegghe is auteur en medeauteur van meerdere wetenschappelijke publicaties. Ze gaf lezingen op internationale en nationale congressen en begeleidde ook enkele eindwerken.

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2011-2012: Moleculaire karakterisering van methicilline resistente *Staphylococcus aureus* isolaten afkomstig varkensvlees. **Kaat Luyckx**

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