Els M. Broens

Livestock-associated methicillin resistant *Staphylococcus aureus* in pigs - prevalence, risk factors and transmission dynamics

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Livestock-associated methicillin resistant *Staphylococcus aureus* in pigs –
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ABSTRACT

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In 2004, an association between human carriage of methicillin resistant *Staphylococcus aureus* (MRSA) and contact with pigs was found. To assess the implications of this finding for veterinary and public health more insight into the prevalence, risk factors and transmission dynamics of this so-called livestock-associated (LA-)MRSA was needed. Therefore, field and experimental studies were conducted in pig and human populations of which the results are presented in this thesis. First, observational studies on pig farms were performed to estimate the prevalence of LA-MRSA positive herds, and to identify factors associated with LA-MRSA in pig herds. It was shown that LA-MRSA was present in the majority, i.e. ~70%, of Dutch pig herds and that the prevalence increased over time. Larger herds were more often found LA-MRSA positive than smaller herds, and transmission was shown to occur by animal trade. From all this, it was concluded that LA-MRSA has become endemic in the Dutch pig population. Secondly, studies on LA-MRSA in pigs, the environment and personnel in pig slaughterhouses were performed. In pigs, a clear increase in LA-MRSA positive pigs from 0 to 60% was shown in the time period between loading at the farm and stunning at the slaughterhouse. This indicated a very rapid transmission of LA-MRSA between pigs through direct contact or through contact with a contaminated environment. An increase in LA-MRSA positive environmental samples taken in the slaughterhouse was found during the working day. In personnel, LA-MRSA prevalence was 6% and working with live pigs was the single most important factor for being positive; personnel not working with pigs or working only with dead pigs were all LA-MRSA negative. Thirdly, transmission of LA-MRSA within herds was studied longitudinally both in an experimental setting and in 6 pig herds. Transmission rates and the factors affecting these rates were determined. The results of both studies indicated that LA-MRSA is able to spread easily and persist in pig populations, resulting in an endemic situation. Use of selective antimicrobials has a positive effect on the transmission rate of LA-MRSA, but transmission occurs even without use of antimicrobials. The key to limiting LA-MRSA transmission from pigs to humans is to eliminate the source, i.e. eradicate LA-MRSA from pig herds, and a combination of different intervention strategies controlling both within- and between-herd transmission will be needed to achieve this.

VOORWOORD

In april 2007 besloot ik mijn baan op te zeggen, even een adempauze te nemen en tijdelijk als verhuiscoördinator van ons gezin van de Noordoostpolder naar de Betuwe aan de slag te gaan. Ware het niet dat mijn oog viel op een interessante vacature als AIO, wat resulteerde in allesbehalve een adempauze en …… dit boekje.

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'Als ik jullie niet had, had ik niemand'

Liefs, Els

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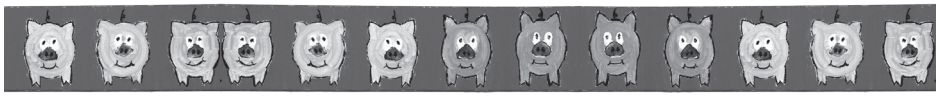
GENERAL INTRODUCTION



GENERAL INTRODUCTION

BACKGROUND

Methicillin resistant *Staphylococcus aureus* (MRSA) is well known for its ability to cause hospital acquired (HA-)infections and outbreaks in hospitalized patients (Grundmann et al., 2006; Klevens et al., 2007). The main reservoir of HA-MRSA is infected or colonized patients within the hospital and from other hospitals (Eveillard et al., 2004; Williams et al., 2009). Worldwide, the prevalence of MRSA among clinical *S. aureus* isolates varies between below 1% and above 40% (Panlilio et al., 1992; Klevens et al., 2006; EARSS, 2007). In Dutch hospitals, this prevalence is low, i.e. < 2% as a result of a strict national search-and-destroy policy (Wertheim et al., 2004a). Known risk factors for introduction in Dutch hospitals are recent hospitalization, admission to an Intensive Care Unit, surgery, exposure to individuals who are colonized or infected with MRSA and prolonged antibacterial therapy (Lucet et al., 2003; Van Belkum et al., 2009).

Since the 1990s, the epidemiology of MRSA has changed. The number of reports on community acquired (CA-)MRSA infections in people with no apparent risk factors is increasing (Chambers, 2001; Kluytmans-Vandenbergh and Kluytmans, 2006). CA-strains are distinct from the traditional HA-strains in genetic background, epidemiology, clinical spectrum and antimicrobial susceptibility pattern (Hiramatsu et al., 2001; Naimi et al., 2003). CA- and HA-lineages are, therefore, different and the origin of CA-strains is divers. As people move between hospitals and the community, so will MRSA strains do as shown by an increase of CA-infections in healthcare settings (Popovich et al., 2008; Webb et al., 2009).

Companion animals and horses were previously identified as potential zoonotic reservoirs for human MRSA infections (Weese et al., 2005; Leonard and Markey, 2008). MRSA in livestock was limited to a case of bovine mastitis in Belgium (Devriese et al., 1972), until human carriage of MRSA was linked to pig farming in 2004 in The Netherlands (Voss et al., 2005). These pig-related strains were not typable by Pulsed Field Gel Electrophoresis (PFGE) with restriction endonuclease *SmaI* (Dutch standard method), and all strains belonged to Clonal Complex (CC) 398 (Bens et al., 2006; De Neeling et al., 2007; Van Loo et al., 2007a). Dutch pilot studies on pig farms and in pig slaughter batches showed pig prevalences of 23% and 81%, respectively (De Neeling et al., 2007; Van Duijkeren et al., 2008). Transmission to humans was confirmed and MRSA CC398 prevalence in humans was found to be associated with the intensity of contact with pigs (Van Loo et al., 2007a; Van Den Broek et al., 2009). After the initial findings in The Netherlands, also other countries reported MRSA CC398 in pigs and people in contact with pigs, indicating an international problem (Guardabassi et al., 2007; Khanna et al., 2008; Smith et al., 2009). Furthermore, case reports appeared on MRSA CC398 in other animal species used for human food consumption, such as veal calves and poultry (Nemati et al., 2008; Graveland et al., 2010; Mulders et al., 2010). MRSA was also found in 11% of retail meat products, but the risk for public health of consuming MRSA positive meat is assumed to be of minor importance (De Boer et

al., 2009). It was concluded that livestock is a zoonotic reservoir for MRSA CC398, now-called livestock-associated (LA-)MRSA.
Since 2006, people in contact with live pigs and veal calves are included in the high-risk group according to the Dutch MRSA 'search-and destroy' policy in hospitals, which implies that they are actively screened for MRSA on hospital admission (WIP, 2007). Similar to other MRSA strains, LA-strains will move back and forth between hospitals and the (farming) community. Invasive infections and nosocomial outbreaks caused by LA-strains have indeed been described (Ekkelenkamp et al., 2006; Declercq et al., 2008; Wulf et al., 2008a; Fanoy et al., 2009), confirming the risk for public health of this identified reservoir.

SCOPE AND OUTLINE OF THIS THESIS

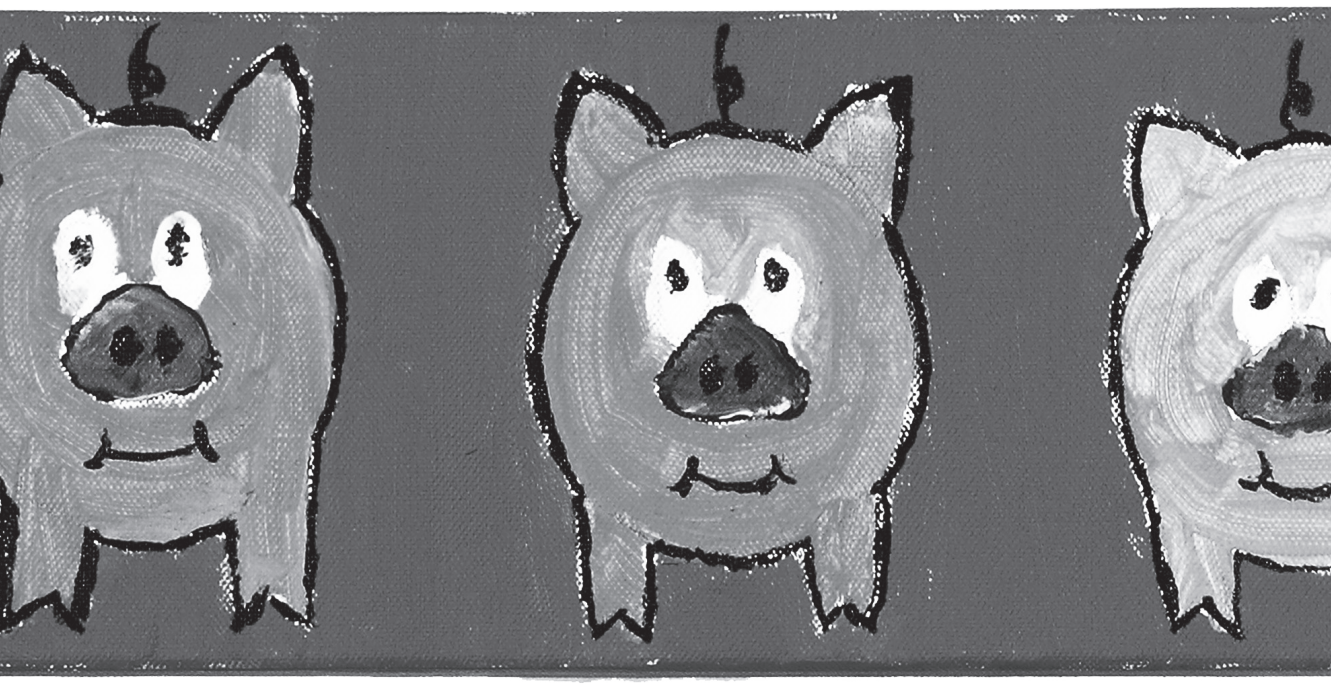
From the livestock reservoir, LA-MRSA can and will be introduced into hospitals and serious infections and outbreaks in humans do occur. In order to identify (cost-) effective intervention strategies in livestock, and with that to prevent the occurrence of infection in humans it is essential to gain more insight in transmission dynamics by studying the transmission routes and rates between animals, from animals to humans and from humans to humans as well. For this, cooperation between experts in epidemiology and microbiology in both human and veterinary medicine is necessary. Therefore, a Dutch MRSA research program was initiated and performed including several research groups from institutes working in the human and veterinary field (Wagenaar and Van De Giessen, 2009). Observational studies focusing on pig populations within the MRSA research program were performed as part of this thesis. Additionally, studies on transmission dynamics of LA-MRSA in pig populations were initiated and designed based on preliminary results from above mentioned observational studies, and described in this thesis as well.
The main aim of this thesis was to gain more insight in the occurrence and transmission dynamics of LA-MRSA in pig populations. For this purpose, field and experimental studies were conducted to answer key questions on (1) prevalence of LA-MRSA on different kinds of pig farms, (2) risk factors for introduction and persistence of LA-MRSA in pig populations and in the pig production chain, (3) transmission between pigs within a herd as well as transmission between herds, and (4) the role of pig populations in the transmission to humans. For this purpose, quantitative data were collected in cross-sectional and longitudinal field studies and in transmission experiments. The results of these studies are presented in Part II to IV of this thesis.
Part I is an introductory chapter *(Chapter 1.1)* and reviews the history and evolution of MRSA, focusing on transmission of MRSA from animals to humans. Knowledge gaps present at the time this chapter was published (2008) are identified and the need for collaboration between experts from different research areas to fill these gaps, is emphasized.
Part II consists of 3 chapters and describes results of several observational studies on LA-MRSA in the pig production chain to study the transmission between farms.

In *Chapter 2.1*, three sampling methods for MRSA classification of pig herds are compared. After the initial Dutch findings, several countries started surveys on LA-MRSA using different sampling methods and laboratory techniques. As one of our studies *(Chapter 2.3)* was carried out simultaneously with an EU-wide survey (EFSA, 2009), we were able to compare different sampling methods for MRSA classification of pig herds. Subsequently, pig farms in all levels of the pig production pyramid were sampled to investigate the role of animal trade as a transmission route for LA-MRSA between herds *(Chapter 2.2)*. To estimate the Dutch national prevalence and quantify risk factors for introduction or persistence of LA-MRSA an observational study on 202 pig farms was performed *(Chapter 2.3)*.

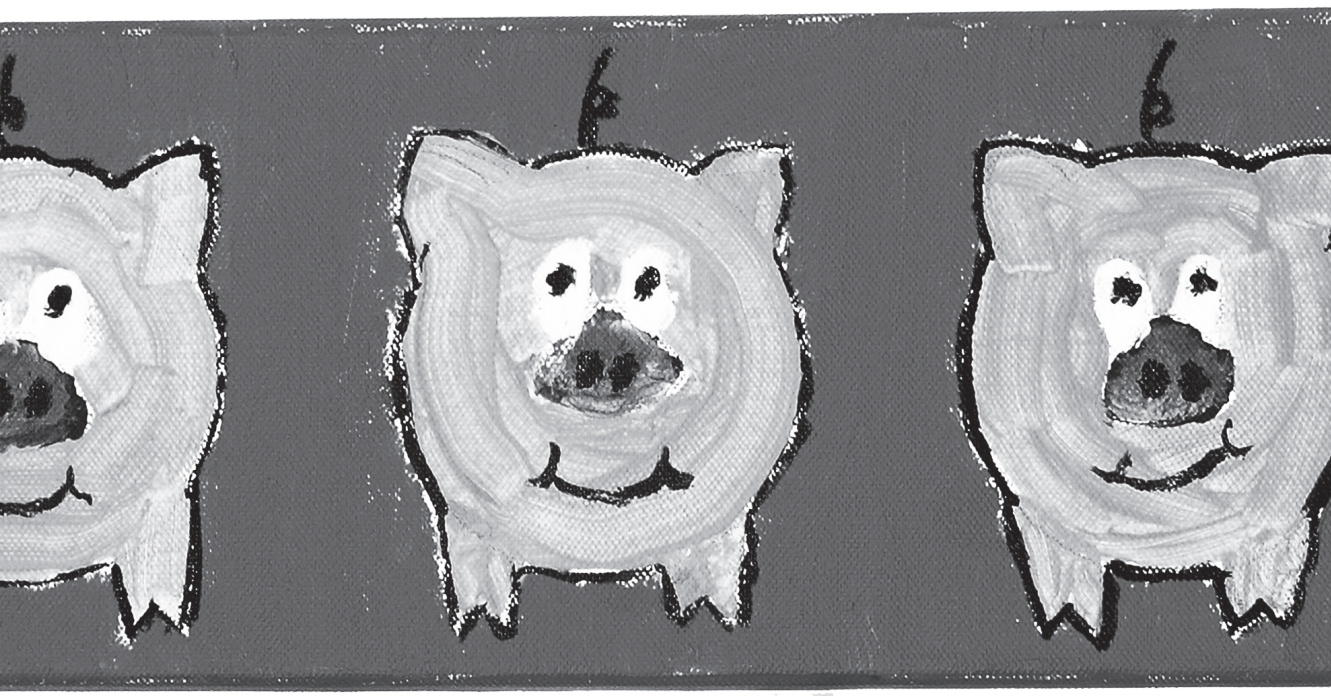
Part III consists of 2 chapters and describes studies on LA-MRSA in the slaughterhouse focusing on both transmission between animals and transmission from animals to humans. To gain more insight into the transmission between animals, it was investigated whether MRSA negative pigs can become MRSA positive during transportation from the farm to the slaughterhouse after exposure to other pigs and environmental sources of MRSA *(Chapter 3.1)*. To gain more insight into the transmission from animals to humans, MRSA prevalence and risk factors for pig slaughterhouse workers in different slaughterhouse sections were examined *(Chapter 3.2)*.

Part IV consists of 2 chapters and focuses on rates of transmission and transmission dynamics of LA-MRSA between pigs within pig populations. First, a transmission experiment was performed to quantify the transmission rate of LA-MRSA between pigs under controlled conditions *(Chapter 4.1)*. Secondly, transmission was studied under field conditions. Longitudinal data from pig farms in Denmark and The Netherlands were collected to estimate the reproduction ratio, which is a quantitative measure of transmission between animals *(Chapter 4.2)*.

Finally, the results presented in the previous chapters are summarized and integrated together with recent information on this topic in the General Discussion. The information is used to evaluate the relevance and implications of the results for veterinary and human medicine and potential intervention and control strategies are discussed.

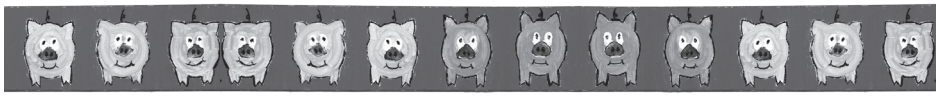


PART I – INTRODUCTION TO LA-MRSA



CHAPTER 1.1

TRANSMISSION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* FROM FOOD PRODUCTION ANIMALS TO HUMANS: A REVIEW



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KEYWORDS

- Antibiotic resistance
- Methicillin resistance
- *Staphylococcus aureus*
- Zoonoses
- Animals
- Evolution
- Transmission

ABSTRACT

International surveillance of antimicrobial use in food animal production shows that methicillin resistant *Staphylococcus aureus* (MRSA), traditionally a human pathogen associated with hospitals, has emerged in the community and animals. Since 1961, MRSA has been causing human infections in hospitals worldwide and a vast majority of them were caused by five major epidemic clones. After 1990, other clones have emerged in the community, leading to infections in relatively young and healthy individuals. The origin of these clones is largely unknown, and extensive diversity among isolates exists. Companion animals have been indicated as a reservoir. However, most studies suggest that they are initially infected by humans and subsequently animals re-colonize humans. More recently, a new zoonotic reservoir in food production animals was found. This involves a specific clone, MRSA ST398, which spreads extensively in animals and is also found in retail meat. It poses a potential threat to public health, as people in contact with food production animals are at much higher risk of colonization. The most probable transmission route seems to be by (in)direct contact, as dust in stables was found positive for MRSA ST398. The role of MRSA ST398 as a food pathogen needs more research. To prevent colonization in humans, it is important to investigate transmission routes and transmission dynamics between animals, between animals and humans and between humans. Collaboration of human and veterinary epidemiologists and microbiologists is needed to identify the implications of this strain for public health and to develop cost-effective control strategies.

Review Methodology:
A search in PubMed and CAB Abstracts without date restriction was performed using the major subheadings 'drug resistance, bacterial', 'methicillin resistance', *'Staphylococcus aureus'*, 'evolution', 'animals', 'MRSA' and 'food animal production' searched in free text. Additional articles were obtained by searching the citations within the retrieved papers. Internet searches were done to obtain data from the European Union, World Organisation for Animal Health (OIE), Centres for Disease Control and Prevention (CDC) and (inter)national surveillance programmes on antimicrobial use and resistance. Furthermore, the topic was discussed with colleagues and information from ongoing studies was incorporated.

INTRODUCTION

Antimicrobial resistance is a public health issue of growing concern. The use of antimicrobials can lead to development of antimicrobial resistance in bacterial species (Tenover and McGowan, 1996; Acar and Rostel, 2001). Antimicrobial use in food animal production may become a public health issue when resistant organisms or their resistance genes spread from animals to humans by (in)direct contact or through the food chain (Aarestrup, 2005; Wassenaar, 2005). Surveillance of antimicrobial resistance in human and veterinary pathogenic and indicator bacteria intends to reveal trends in the evolution of resistant organisms (Hammerum et al., 2007). An important, traditionally human pathogen, methicillin resistant *Staphylococcus aureus* (MRSA) is currently causing a pandemic in hospitals around the world and is also emerging in the community (Chambers, 2001). For example, in the USA, MRSA was responsible for an estimated 94 000 life-threatening infections and 18 650 deaths in 2005, which is more than the 16 268 deaths caused by HIV/AIDS in that same period (Klevens et al., 2007; CDC, 2008). Recently, MRSA has been identified in food production animals and people in contact with these animals (Voss et al., 2005). This involves a specific clone, Multi Locus Sequence Type 398, which seems to spread extensively among animals (Graveland et al., 2008; De Neeling et al., 2007; Khanna et al., 2008; Van Den Eede et al., 2009). The finding of this new zoonotic reservoir of MRSA has led to several research initiatives to investigate its implications. This paper intends (1) to describe the evolution of MRSA in general and specifically of the ST398 strain and (2) to review the transmission between animals and humans in order to assess its impact on veterinary and public health.

ANTIMICROBIAL RESISTANCE AND FOOD ANIMAL PRODUCTION

Resistance of bacteria to a particular antimicrobial agent can be mediated by a pre-existing phenotype in natural bacterial populations or by acquired resistance. Two genetic mechanisms are involved in acquiring and disseminating resistance: (1) de novo mutations or (2) horizontal transmission of resistance genes between individual bacteria or between bacterial species. Resistance acquired through either mechanism is subsequently transmitted, and the frequency of resistance in populations may increase as a result of selective advantage under the pressure of antimicrobial use (Acar and Rostel, 2001; Tenover, 2006).

Antimicrobial agents are widely used in humans, animal husbandry and other agricultural activities. Since any use of antimicrobial agents can result in the selection for resistance, antimicrobial usage in animals has contributed to the development of resistance in bacterial species (Aarestrup and Wegener, 1999; Van Boven et al., 2003; Aarestrup, 2005). Transmission of resistant bacteria from farm animals to humans can occur not only by (in)direct contact, but also through food products of animal origin (Kluytmans et al., 1995; Bywater et al., 2004; Eveillard et al., 2004). If these are zoonotic pathogens, this can lead to human disease with potential treatment failure. Additionally, resistant bacteria can trans-

fer their resistance genes to other bacteria belonging to human commensal flora (Acar and Rostel, 2001; Aubry-Damon et al., 2004).

Modern food animal production has high production levels, high stock densities and small profit margins. An environment has been created where infectious disease can have disastrous consequences. Rapid dissemination of pathogens is facilitated by the high contact rate between animals and by animal transport. To prevent outbreaks of infectious diseases, hygiene measures are being improved, management is optimized and vaccines are applied. Despite all these interventions, the use of antimicrobials in food production animals is often inevitable. Antimicrobials are not only used to treat diseases, but antimicrobials are also applied strategically to prevent infections and, in several parts of the world, as growth promoters (Van Den Bogaard and Stobberingh, 2000; Aarestrup, 2005; Wassenaar, 2005).

Although the evidence for resistant bacterial infections in humans as a consequence of antibiotic use in food producing animals is sparse, there is a general belief that prudent use of antimicrobials in food animal production should be given high priority. In 1969, the Swann Committee had already made this recommendation to the British government (Swann, 1969). Only after increasing reports of resistant zoonotic human infections, antimicrobial growth promoters (AGPs) were phased out in Sweden, Norway and Denmark in 1986, 1995 and 1998–99, respectively, followed by a total ban from January 2006 onwards in all countries of the European Union (Phillips, 2007). Experiences in the Scandinavian countries are promising; despite an initial increase in antimicrobial use for therapeutic reasons, the total use of antimicrobial agents declined substantially after the ban on AGPs (Wierup, 2001; Bengtsson and Wierup, 2006; Grave et al., 2006).

The increased attention being given to antimicrobial use in food animal production has led to the creation of several national and international surveillance systems (MARAN, 2006; DANMAP, 2007; EARSS, 2007; NORM/MORM-VET, 2007; NARMS, 2008; SVARM, 2008). In general, these surveys collect and present yearly data on usage of antimicrobial agents and the occurrence of resistance in bacteria from animals, food and humans. Most surveys discriminate between three groups of bacteria: (1) indicator bacteria, e.g. enterococci and *Escherichia coli*, (2) zoonotic bacteria, e.g. *Salmonella* spp. and *Campylobacter* spp., and (3) human pathogenic bacteria, e.g. staphylococci and streptococci. A finding in these reports is that MRSA, traditionally a human pathogen associated with hospitals, has emerged more and more into the community and also into animals in the last decades, which implicates a new zoonotic reservoir of MRSA (Mevius and Verbrugh, 2006; DANMAP, 2007; EARSS, 2007; Hammerum et al., 2007).

COLONIZATION AND INFECTION WITH *S. AUREUS*

S. aureus is a Gram-positive, coagulase-positive coccus in the family Staphylococcaceae. Staphylococcal species occur worldwide as commensal colonizers of the skin of animals and humans. They are additionally found on mucous membranes of the upper respiratory tract and lower urogenital tract and transiently in the

digestive tract. Staphylococci are resistant to dehydration and are stable for months in the environment (Quinn et al., 2002).

It is important to note that a distinction must be drawn between colonization and infection by *S. aureus* and its methicillin resistant variant. Colonization with *S. aureus* may occur on mucous membranes of the respiratory and/or intestinal tract, or on other body surfaces, without causing disease or harming their hosts (Scanvic et al., 2001; Eveillard et al., 2004). Some individuals are colonized transiently and some persistently (Kenner et al., 2003). Colonization with *S. aureus* usually precedes infection, and is mostly caused by the same subtype (Casewell, 1998; Wertheim et al., 2004b). The prevalence of nasal colonization with *S. aureus* among the human population is relatively high (> 24%), while the prevalence of nasal colonization with MRSA among the same group is low (< 1.5%) (Wertheim et al., 2004a; Gorwitz et al., 2008). When the opportunity arises, *S. aureus* can contaminate wounds, bloodstream or other tissues, causing serious and even life-threatening infections (Quinn et al., 2002; Begier et al., 2004; Ellis et al., 2004; Morris et al., 2006; Noskin et al., 2007). A US study on *S. aureus* infections in patients reported an increase in prevalence from 0.74% in 1998 to 1.0% in 2003 (Noskin et al., 2007).

Infected and colonized human individuals constitute a major reservoir of *S. aureus*, and the primary route of *S. aureus* transmission seems to be direct contact with infected or colonized individuals (Eveillard et al., 2004; Boyce, 2007; Albrich and Harbarth, 2008). However, environmental spread may be a substantially underestimated route for *S. aureus* transmission in hospitals (Henderson, 2006; Sexton et al., 2006).

FROM *S. AUREUS* TO MRSA

Soon after the introduction of methicillin in 1961, the first MRSA was described (Jevons et al., 1963). This resistance is present when *S. aureus* has acquired the *mecA* gene, which codes for a variant of the penicillin-binding protein (PBP), PBP2a. PBP, normally present at the cell membrane of *S. aureus*, is bound by penicillin, and consequently cell membrane synthesis is discontinued, resulting in bacterial death. However, PBP2a has a reduced affinity for β-lactam antibiotics, leaving the cell membrane intact and the organism alive (Pinho et al., 2001).

The *mecA* gene resides on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito et al., 2001). SCC*mec* contains a *mec* complex, which includes the *mec*A gene and one or two regulatory genes, and a cassette chromosome recombinase *(ccr)* gene complex, which regulates the insertion and excision of the cassette into the bacterial chromosome. So far, five different *mec* complexes and three different *ccr* genes have been described, combining into five different SCC*mec* types (Katayama et al., 2000; Ito et al., 2001, 2004).

The origin of the SCC*mec* element is unknown. SCC*mec* elements have been described in methicillin resistant coagulase-negative staphylococci, although not in other genera. A homologue of the *mecA* gene is found in the species *Staphylococcus sciuri*, which occurs in animals. Most likely the *mecA* gene evolved out

of a recombination of a normal PBP gene and an inducible β-lactamase gene (Wu et al., 2001; Deresinski, 2005). The most acceptable theory is that the *mec* and *ccr* genes were combined in coagulase-negative staphylococci (most likely *Staphylococcus epidermidis*). Subsequently, a deletion in the *mec*-regulatory genes took place and the SCC*mec* complex was acquired by a methicillin susceptible *S. aureus* (MSSA), creating the first MRSA (Oliveira et al., 2002; Grundmann et al., 2006; Deurenberg et al., 2007).

THE EVOLUTION OF MRSA

The investigation of the evolution of MRSA relies on typing methods as tools for the characterization of and the distinction between different isolates. The historically applied phenotypic methods have their limitations and are now out of favour because of newly developed genotypic methods, which usually provide better discriminatory power (Maslow et al., 1993; Faria et al., 2008).

Molecular typing methods

S. aureus isolates, including MRSA, can be typed using several molecular methods. The most important typing methods include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), SCC*mec* typing and staphylococcal protein A typing (*spa* typing). The most appropriate method to use depends on the intended purpose; a combination of different techniques is often used (Cookson et al., 2007; Faria et al., 2008).

For PFGE, genomic bacterial DNA is digested using *Sma*I (a restriction enzyme). Different strains are typed by comparing the presence and length of digestion products using gel electrophoresis. PFGE is a highly discriminatory method, and is very useful in outbreak situations, but reproducibility and inter-laboratory agreement are insufficient to be useful for long-term epidemiological surveillance (Tenover et al., 1995; Cookson et al., 2007; Melles et al., 2007).

With MLST, the allelic profile of seven housekeeping genes can be summarized in a sequence type (ST), a group of strains with seven identical loci. These housekeeping genes are highly conserved, generating a method very useful in the study of clonal evolution (Enright and Spratt, 1999; Enright et al., 2000; Robinson and Enright, 2004). With 'based upon related sequence types' (BURST) analysis, different STs can be grouped; all the strains in one clonal complex (CC) share at least five out of seven housekeeping genes. The CC is numbered after the ST that gave rise to it, the clonal ancestor. Single locus variants (SLVs) are strains with only one locus different from its ancestor (Feil et al., 2004; details available from http://www.mlst.net). MLST provides a combination of discriminatory power and clonal stability, which makes its results unambiguous and easily exchangeable between laboratories (Enright and Spratt, 1999; Trindade et al., 2003).

The AFLP technique is based on the amplification of subsets of genomic restriction fragments using polymerase chain reaction (PCR) and documents nucleotide sequence variation, insertions and deletions across the entire genome. Results of

AFLP are easy to obtain and highly reproducible, and the obtained typing patterns seem to cluster according to the SCC*mec* types of the strains (Vos et al., 1995; Van Der Zee et al., 2005; Melles et al., 2007).
Typing of the SCC*mec* element is done in various ways. The most appropriate method has not yet been determined (Oliveira and De Lencastre, 2002; Zhang et al., 2005; Chongtrakool et al., 2006; Faria et al., 2008).
In *spa* typing, one single locus (staphylococcal protein A) is sequenced, making interpretation of the results very simple and exchangeable, also internationally. The discriminatory power of *spa* typing is in between PFGE and MLST. Therefore, it is suitable for both outbreak situations and evolutionary studies (Frenay et al., 1996; Harmsen et al., 2003; Malachowa et al., 2005).

Molecular evolution of MRSA

Two theories exist on the molecular evolution of MRSA. The single clone theory states that all MRSAs have one common ancestor; the SCC*mec* element was introduced into SA only once (Kreiswirth et al., 1993). The multiclone theory states that SCC*mec* was introduced multiple times in different SA lineages, after which horizontal spread and recombination were important mechanisms of resistance transmission (Musser and Kapur, 1992; Feil et al., 2003). Using the different techniques stated above, the theories have been tested and the following can be concluded: (1) prevalent MSSA strains that were successful in causing disease on a global scale have evolved into MRSA on multiple, but independent occasions, (2) horizontal transfer of the SCC*mec* has occurred a limited number of times compared with other bacteria and (3) clonal spread after acquiring the SCC*mec* appears to be the most important mechanism of dissemination of resistance (Kluytmans, 1993; Enright et al., 2002; Robinson and Enright, 2004; Deurenberg et al., 2007).
Since the first human MRSA was isolated in 1961, at least five major clonal types (CC8, CC5, CC45, CC22 and CC30) of MRSA have been identified using PFGE (Robinson and Enright, 2003; Stefani and Varaldo, 2003; Aires De Sousa and De Lencastre, 2004). The five types predominantly harbour SCC*mec* type I, II or III and are often multidrug-resistant (Enright, 2003). These clones are responsible for the vast majority of MRSA infections in hospitals all over the world. MLST revealed two distinct ancestors for these five, so-called, epidemic clones (Oliveira et al., 2002).
Besides the epidemic clones, there are also clones that occur only in single hospitals or even only in single patients (sporadic isolates) and isolates that cause infections in the community (community-acquired (CA) isolates) (Aires De Sousa and De Lencastre, 2003, 2004; Fey et al., 2003; Hallin et al., 2008). The characterization of these clones revealed extensive diversity among isolates. Several studies observed strong similarities between sporadic isolates and CA-MRSA, which implies that MRSA strains described as CA may actually originate from hospitals (Aires De Sousa and De Lencastre, 2003, 2004; Enright, 2003). CA-MRSA isolates frequently carry SCC*mec*-IV or V, are susceptible to a limited number of antimicrobials and may contain additional virulence factors (Okuma et al., 2002;

Fey et al., 2003; Naimi et al., 2003). SCC*mec* IV and V are much smaller than SCC*mec* I, II and III, which may lead to a more efficient transfer of the element between bacteria and less fitness cost in everyday metabolism (Ito et al., 2001, 2004; Robinson and Enright, 2003; Deresinski, 2005). With this greater ability for transmission and virulence, MRSA clones in the community might be an even larger threat to patients and health-care workers than hospital-acquired clones. Until now, resistance to more than just β-lactam antibiotics is relatively infrequent in these community clones, but future mutations or gene transfer may change this (Okuma et al., 2002; Henderson, 2006).

Just a few studies have been done on molecular characterization of (MR)SA in animals, which hampers the investigation of their relatedness and origin. The fact that *S. aureus* is found in different animal species makes it even more difficult, since information on (MR)SA in one species might not be comparable with information on (MR)SA in another species. Several human MLST types have been found in companion animals, suggesting interspecies transmission (Armand-Lefevre et al., 2005; Malik et al., 2006; Weese et al., 2006; Monecke et al., 2007). Recently, an MRSA strain was detected that appeared not typable by PFGE, owing to resistance to *Sma*I digestion (Bens et al., 2006). These isolates predominantly originate from livestock and are found in humans as well. This strain belongs to MLST type 398 and harbours SCC*mec* IV or V, is mostly associated with *spa* types t034, t011, t108, t567, t571 and t899 and does not contain toxin genes (Voss et al., 2005; Huijsdens et al., 2006; De Neeling et al., 2007; Van Loo et al., 2007a; Van Duijkeren et al., 2008). Different antibiotic resistance patterns have been found, which seem to be correlated with the antibiotics used in animal practice, predominantly tetracyclins (De Neeling et al., 2007; Van Duijkeren et al., 2008). MRSA ST398 might originally have been a highly prevalent strain of MSSA among livestock that acquired *mecA* from other staphylococci that colonize pigs (Aubry-Damon et al., 2004; Armand-Lefevre et al., 2005). The fact that several different SCC*mec* elements have been found in MRSA ST398 suggests that this event must have occurred on several occasions.

MRSA IN HUMANS

Infections in humans with so-called hospital-acquired (HA)-MRSA were confined to patients with established risk factors, including recent hospitalization, admission to an Intensive Care Unit, surgery, exposure to individuals who are colonized or infected with MRSA and prolonged antibacterial therapy (Troillet et al., 1998; Papia et al., 1999; Lucet et al., 2003). However, a modelling study showed that in low-prevalence countries, outbreaks of HA-MRSA might also be initiated by strains that circulate (without clinical signs) among the general population (Bootsma et al., 2006).

Worldwide, clinical cases of MRSA are increasing over the years. In the USA, MRSA prevalence among SA isolates in hospitals has increased from 2.4% in 1975 to 29% in 1991 (Panlilio et al., 1992). Between 1992 and 2003, the percentage of SA isolates from patients in US Intensive Care Units that were methicillin resistant

rose from 35.9 to 64.4% (Klevens et al., 2006).

In England and Wales, the percentage of SA bacteraemia caused by MRSA increased from 1–2% in 1990–1992 to approximately 40% in 2000 (Johnson et al., 2001). The European Antimicrobial Resistance Surveillance System (EARSS) monitors antimicrobial resistance continuously in most European countries. The proportion of methicillin resistance in invasive SA isolates varies largely across Europe, with the highest proportions (> 40%) in Southern Europe and parts of Western Europe and the lowest proportions (< 1%) in Northern Europe. MRSA in low-prevalence countries (< 3%) was most frequently related to patients with a recent history of being admitted in a foreign hospital. The prevalence of MRSA in clinical isolates in these low-prevalence countries remained relatively stable over time. However, since 1999 small increases were found in The Netherlands (0.34–0.93%), Denmark (0.28–1.70%) and Finland (0.95–2.91%) (EARSS, 2007).

In the last two decades, several reports appeared about MRSA infections in healthy individuals in the community without health-care-associated risk factors (Chambers, 2001; Naimi et al., 2003; Kluytmans-Vandenbergh and Kluytmans, 2006). Such infections are referred to as CA and are distinct from HA-MRSA infections in terms of genetic background, epidemiology, clinical spectrum and antibacterial resistance (Hiramatsu et al., 2001; Naimi et al., 2003). Several definitions have been proposed, but no standard definition has been created for CA-MRSA yet, which makes the overall prevalence of CA-MRSA hard to ascertain (Salgado et al., 2003). However, according to several studies, MRSA infections in healthy people without the established risk factors seem to be increasing in frequency (Herold et al., 1998; Bukharie et al., 2001; Drews et al., 2006). Children, elderly people and people in groups with close physical contact, e.g. US football teams and military recruits, seem to be at risk (Shahin et al., 1999; Borer et al., 2002; Begier et al., 2004; Zinderman et al., 2004; Nguyen et al., 2005; Beam and Buckley, 2006). Food-borne infections occur as well, where the source might be the food animal itself or the food-processing person or equipment (Kluytmans et al., 1995; Jones et al., 2002; Lee, 2003; Normanno et al., 2007).

Current control strategies in countries where the spread of MRSA is still under control, such as The Netherlands and Scandinavia, have focused on the health-care sector and are based on active surveillance for detection of MRSA carriers among patients ('search'), and isolation of colonized and infected patients and decolonization therapy ('destroy') (Muto et al., 2003; Struelens and Denis, 2006). Decolonization consists of topical and systemic administration of antimicrobials, and although protocols have been developed that can eradicate MRSA, re-colonization can occur (Kniehl et al., 2005; Simor et al., 2007). As MRSA is able to survive for weeks or months in the environment, decontamination is a very important component of the control strategy (Kniehl et al., 2005; Sexton et al., 2006; Boyce, 2007). In the low-prevalence countries, this so-called search-and-destroy strategy prevents MRSA from becoming endemic (Wertheim et al., 2004a). However, the same strategies have failed in the past in countries where MRSA is endemic at a high level (Marshall et al., 2004). Recent findings suggest that effective control should be possible by stringent implementation of this search-and-destroy

strategy, even in countries where MRSA already is an important nosocomial pathogen (Bootsma et al., 2006).
The increasing frequency of MRSA infections in the community implies an adjustment of the guidelines for the 'search' part of existing control strategies as the 'at risk' groups are expanding.

MRSA IN ANIMALS

The isolation of MRSA from animals was first reported in 1972 following its detection in milk from mastitic cows (Devriese et al., 1972). Since then, MRSA has been isolated from many different animal species, including dogs (Van Duijkeren et al., 2004a), cats (Morris et al., 2006), horses (Weese et al., 2005), sheep (Goni et al., 2004), pigs (Voss et al., 2005), dairy cows (Juhasz-Kaszanyitzky et al., 2007), veal calves (Graveland et al., 2008; Mooij et al., 2007) and poultry (Lee, 2003; Leenders et al., 2007; Nemati et al., 2008). The increasing number of publications on MRSA in, mainly, companion animals and horses was reviewed by Leonard and Markey (Leonard and Markey, 2008) and they suggest that MRSA may be an emerging pathogen in these species. However, data on MRSA in food production animals were not reviewed. A distinction should be made between food production animals, which are housed in an industrialized way at high stocking densities, and animals that are, predominantly, kept for companionship and leisure purposes.
In food production animals, a new strain of MRSA (ST398) turned up recently (Huijsdens et al., 2006; Leenders et al., 2007; Mooij et al., 2007), whereas companion animals most often are colonized or infected with classical human strains of MRSA (Manian, 2003; Van Duijkeren et al., 2004b; Loeffler et al., 2005).
In pigs, MRSA ST398 seems to spread extensively. Dutch studies report prevalences of positive farms varying from 23 to 81%, whereas the prevalence in individual pigs varies from 11 to 39% (Broens et al., 2008a; De Neeling et al., 2007; Van Duijkeren et al., 2008). After the initial findings in The Netherlands (Voss et al., 2005; Huijsdens et al., 2006; De Neeling et al., 2007), also Belgium (Dewaele et al., 2008), Denmark (Guardabassi et al., 2007), Germany (Meemken et al., 2008), France (Armand-Lefevre et al., 2005), the USA (Smith et al., 2008) and Canada (Khanna et al., 2008) reported the occurrence of MRSA in pigs.
In veal production, high prevalences of MRSA ST398 were found as well: 88% of the farms and 28% of the calves tested positive (Graveland et al., 2008). Furthermore, a human case report on MRSA ST398 linked to poultry and the finding of MRSA ST398 in SA isolates from poultry has been published (Leenders et al., 2007; Nemati et al., 2008). This, however, has not yet been confirmed in larger surveys.
Studies on risk factors for MRSA infections in animals have been performed for small animals and horses admitted to veterinary hospitals. Similar to the factors in human cases, prolonged hospitalization, surgery, contact with MRSA colonized individuals and antimicrobial use were found to be significant factors (Duquette and Nuttall, 2004; Pfeiffer et al., 2005; Weese and Lefebvre, 2007). Preliminary results of studies on pig farms reveal that farms where finishing pigs are present

were more often MRSA positive than farms without finishing pigs and that MRSA seems to spread downwards through the pig production chain by the purchase of positive pigs (Broens et al., 2008a, 2008b; Van Duijkeren et al., 2008). Few studies have investigated dust inside pig stables. In these studies a strong association between results from animal samples and environmental samples was found, which might indicate the possibility of spread of MRSA ST398 between animals within a farm by air (Broens et al., 2008a; Dewaele et al., 2008). However, so far no controlled studies on population dynamics and risk factors for MRSA in farm animals have been published.

Just a few studies on eradication and control of MRSA in pets and horses have been conducted (Duquette and Nuttall, 2004; Weese and Rousseau, 2005). The British Small Animal Veterinary Association has drawn up guidelines on how to manage MRSA (available from http:// www.bsava.com). These are all control measures and guidelines for individual veterinary hospitals and practices. No studies, however, have been conducted to test these strategies on livestock farms so far.

TRANSMISSION BETWEEN HUMANS AND ANIMALS

The role of animal populations in the transmission of pathogens to humans is obligatorily dependent not only on the possibility of transmission from animals to humans, but also on the possibility of transmission between animals.

Dogs and cats were initially indicated as a reservoir for MRSA years ago (Scott et al., 1988; Cefai et al., 1994). The strains involved in canine and feline cases are usually similar to those infecting humans, and the most obvious explanation for this veterinary problem is that the pets acquired the resistant bacteria from humans and that these strains can also be passed back from animals to humans (Van Duijkeren et al., 2004b; Loeffler et al., 2005; Pfeiffer et al., 2005). This does not necessarily imply that companion animals are a reservoir.

In several studies, MRSA was isolated from horses and horse personnel. All the isolates appeared to be identical or closely related and differed from common human isolates (Seguin et al., 1999; Weese et al., 2005). Therefore, MRSA in horses has zoonotic potential. However, quantitative risk assessment of this reservoir has not been done.

Several studies on pig farmers showed a significant higher risk for *S. aureus* carriage in this group. Nasal *S. aureus* colonization was found significantly more often in French pig farmers (45%) than in non-farming controls (24%), suggesting transfer of these bacteria from farm animals to farmers (Aubry-Damon et al., 2004; Armand-Lefevre et al., 2005). Dutch pig farmers and veterinarians were screened at different occasions and showed prevalences of MRSA carriage of > 20 and 4.6%, respectively, whereas the prevalence in the general population upon hospital admission in The Netherlands is 0.03% (Wertheim et al., 2004a; Voss et al., 2005; Wulf et al., 2006). Moreover, it was found that the density of MRSA ST398 in a particular area corresponds to the density of pig farming, whereas the density of other MRSAs corresponds to the density of the human population (Van Loo et

al., 2007a). An international study among pig veterinarians revealed an overall prevalence of 12.5%; MRSA carriers originated from nine different countries (Wulf et al., 2008b).

Hospital surveys that investigated the association of non-typable MRSA in human patients with a reservoir in animals identified cattle as another source for human carriage, next to pigs. Carriers of MRSA ST398 were more often people in contact with pigs or cattle than carriers of other strains of MRSA (Van Loo et al., 2007a; Van Rijen et al., 2008). Surveys on Dutch pig farms supported the finding that contact of humans with the animals is an important risk factor for human MRSA colonization. The intensity of contact was strongly associated with increased prevalence. People with intensive contact with pigs were more often MRSA carriers (29%) than people who lived on these farms and had no contact with the animals (2%) (Van Den Broek et al., 2009). The association between intensity of human–animal contact and MRSA prevalence of humans was confirmed on veal farms, although no exact figures have been published yet (Graveland et al., 2008). So far, just a few studies reported clinical infections in humans caused by MRSA ST398 (Ekkelenkamp et al., 2006; Declercq et al., 2008; Witte et al., 2007). Nevertheless, the first hospital outbreak caused by MRSA ST398 has already been described (Wulf et al., 2008a). To investigate the differences between typable and non-typable MRSA, a limited hospital survey was performed. The frequency of an infection caused by MRSA was higher in patients who carried a typable strain than in patients carrying a non-typable strain: 42 and 13%, respectively. For typable MRSA, the number of secondary cases was 22 out of 2139 contact persons (1%), whereas, for non-typable MRSA, no secondary cases were found in the 408 contact persons (Van Rijen et al., 2008). These findings might indicate that transmission between humans is less likely and that this clone is less adaptive for humans than it is for animals.

The presence of MRSA ST398 in environmental swabs indicates that direct contact with animals is not necessarily needed for transmission to humans (Broens et al., 2008a; Dewaele et al., 2008; Van Den Broek et al., 2009). Based on the above-stated findings in pig and veal farming, people who are professionally in contact with live pigs and veal calves are now included in the high-risk group for the Dutch MRSA 'search-and destroy' policy in hospitals (WIP, 2007).

Other food production animals are reported to be zoonotic sources for MRSA as well. A recent Dutch case report suggests that poultry may be a source of human MRSA infection. People living on a poultry farm and chicken droppings from the same farm were positive for an identical MRSA strain (Leenders et al., 2007).

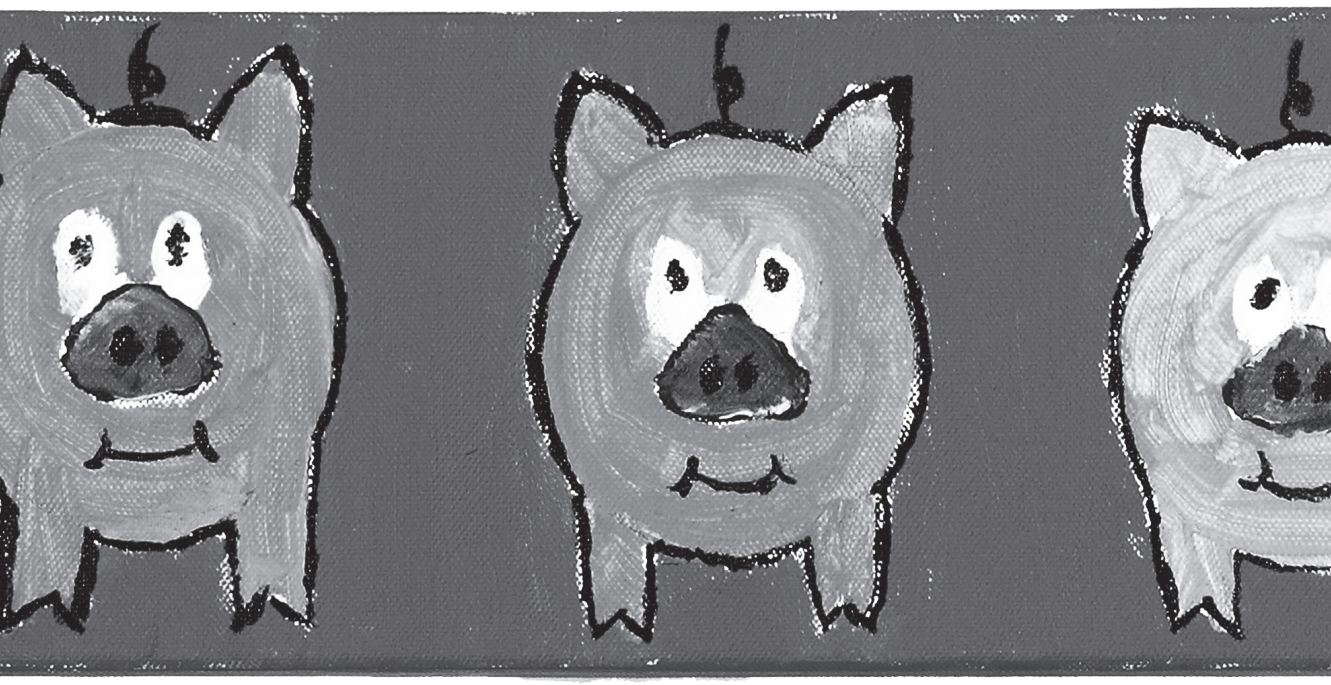
In the past, SA strains isolated from mastitic cows and from human infections were generally found to be different strains (Teale, 2002). However, a recent Hungarian study has found clear evidence that certain MRSA strains can be passed between cattle and humans (Juhasz-Kaszanyitzky et al., 2007).

Studies on MRSA in meat products demonstrate that MRSA originating from animal sources has entered the food chain (Teale, 2002; Lee, 2003; VWA, 2008; Van Loo et al., 2007b). MRSA was found in meat products originating from animals. A recent survey of the Dutch Food and Safety Authority in almost 1300 meat

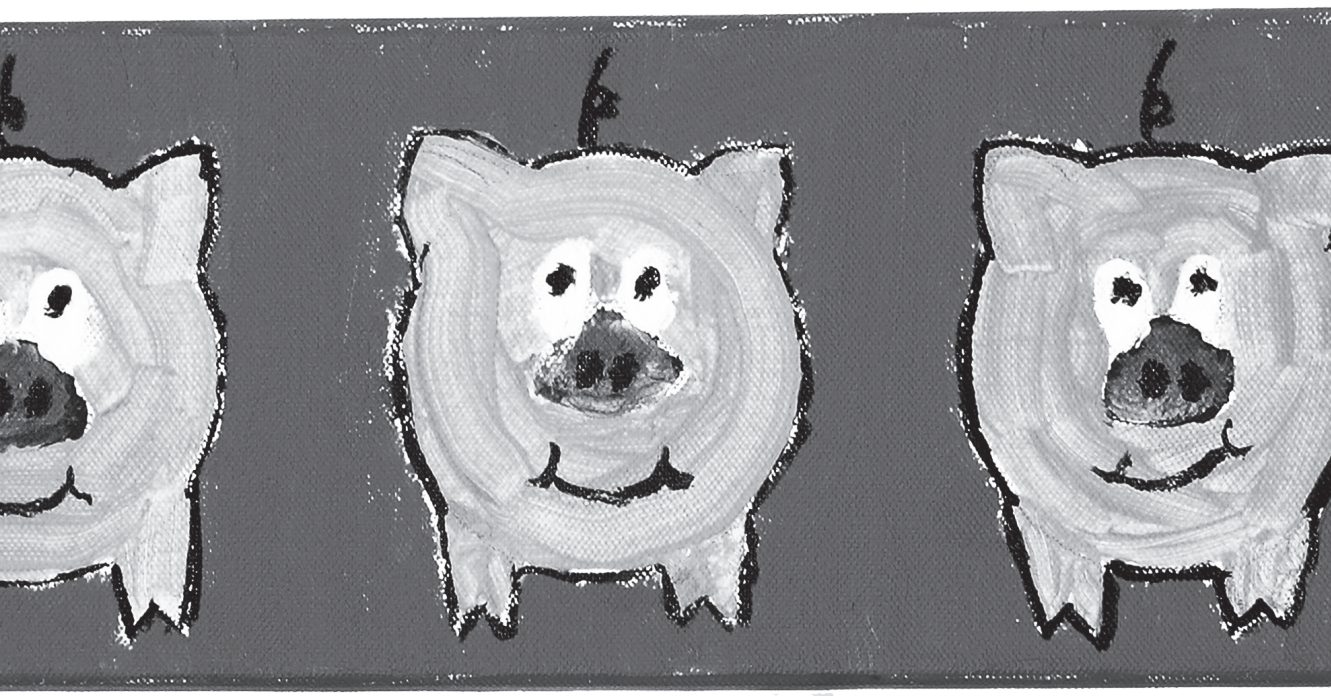
products from retail showed a prevalence of 11%. The highest prevalences were found in products of turkey (31%), chicken (27%) and veal (17%), whereas 10% of pig products were positive (VWA, 2008). So far, the risk for public health of eating MRSA positive meat is assumed to be of minor importance (DANMAP, 2007; VWA, 2008; Van Loo et al., 2007b).

SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

Over the past decades the epidemiology of MRSA has changed significantly. MRSA, traditionally a primarily nosocomial pathogen, has entered the community, causing serious infections. Additionally, MRSA infection and colonization has been documented in several animal species. Although several reports have presented information suggesting that animals may act as a source for zoonotic staphylococcal infections in humans, no transmission studies have been done for MRSA yet.
Recently, MRSA ST398, a novel clone linked to food production animals, has emerged in humans. Molecular typing methods support the relationship between this particular strain in food production animals and humans who have been in contact with these animals. From the animal reservoir, MRSA can be introduced into hospitals and serious infections and outbreaks may occur. Since farm animals are usually housed together in groups, frequent contact between group members is likely. As (in)direct contact is a major route for MRSA transmission between animals, the prevention and control of MRSA in food production animals should focus on the control of the spread of MRSA between animals within a farm and between farms rather than only controlling the organism in individual hosts. In order to design an effective intervention programme for decreasing the risk for public health, experimental and longitudinal research is needed to gain insights into the transmission dynamics of MRSA between animals within a farm and between farms.
Considering the huge spread of MRSA ST398 among food production animals, it is unlikely that this will be eradicated easily. To prevent the occurrence of disease in humans, it is important to investigate the transmission routes from animals to humans and from humans to humans as well. The most probable transmission route seems to be by (in)direct contact, but the role of MRSA as a food pathogen needs more research.
Human microbiologists should investigate the pathogenicity and the capacity for transmission between humans of this particular novel strain to assess the potential threat for public health. At the same time, cooperation between epidemiologists and microbiologists in the human and veterinary field will be required to create a complete overview of all aspects of this problem and to develop cost-effective prevention strategies in both the human and animal populations.

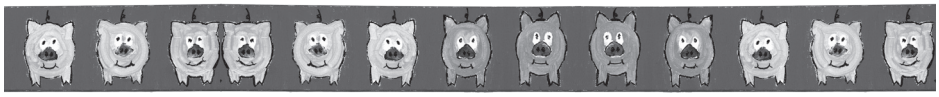


PART II – OBSERVATIONAL STUDIES ON LA-MRSA IN THE PIG PRODUCTION CHAIN



CHAPTER 2.1

COMPARISON OF SAMPLING METHODS USED FOR MRSA CLASSIFICATION OF HERDS WITH BREEDING PIGS



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KEYWORDS

- MRSA
- Pig
- Environment
- Pooling
- Prevalence
- Sampling
- Detection

ABSTRACT

Since the first report on methicillin resistant *Staphylococcus aureus* (MRSA) CC398 in pigs, several countries have determined the prevalence of MRSA positive pig herds using different sampling and laboratory techniques. The objective of the study was to compare three sampling methods for MRSA classification of herds. Therefore, nasal swabs of pigs and environmental wipes were collected from 147 herds with breeding pigs. Per herd, laboratory examination was done on 10 pools of 6 nasal swabs (NASAL), 5 single environmental wipes (ENVSINGLE) and one pool of 5 environmental wipes (ENVPOOL). Large differences in apparent prevalence of MRSA positive herds between methods were found: 19.1% for ENVPOOL, 53.1% for ENVSINGLE, and 70.8% for NASAL. Pairwise comparisons of methods resulted in relative sensitivities of 26.9% (ENVPOOL vs. NASAL), 34.6% (ENVPOOL vs. ENVSINGLE), and 72.1% (ENVSINGLE vs. NASAL) with relative specificities of respectively 100%, 98.6% and 93.0%. Cohen's kappa was respectively 0.18, 0.32 and 0.55, thus varying between very poor and moderate agreement. Examination of environmental wipes is an easy and non-invasive method to classify herds for MRSA. The number of environmental wipes needed depends on e.g. required detection limits and within-herd prevalence. In low prevalent herds (e.g. herds with < 3 positive pools of nasal swabs), 25 single environmental wipes are required to be 90% sure that MRSA is detected at a detection limit similar to analysing 10 pools of nasal swabs. Individual analysis of environmental wipes is highly recommended, as pooling 5 environmental samples resulted in a substantial reduction of the apparent prevalence.

INTRODUCTION

A distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA CC398) has emerged among pigs, veal calves, poultry and people in contact with livestock since 2005 (Graveland et al., 2008; Voss et al., 2005; Mulders et al., 2010). Several countries have determined national prevalences of MRSA positive pig herds and a EU-wide baseline survey on MRSA prevalence in herds with breeding pigs was performed in 2008 (Broens et al., 2008a; Dewaele et al., 2008; Khanna et al., 2008; EFSA, 2009; Smith et al., 2009).

To determine the MRSA status of pig herds, different sampling methods and laboratory techniques are used. A procedure using pre-enrichment in combination with selective enrichment for MRSA detection in nasal swabs from pigs was evaluated as good by Graveland et al. (2009). Several studies have also detected MRSA in dust from inside stables (Broens et al., 2008a; Dewaele et al., 2008; Van Den Broek et al., 2009). In a prior study, Cohen's kappa was 0.68, indicating a good agreement between MRSA classification of 50 pig herds based on the results of either 10 pools of nasal swabs or 5 single environmental wipes (Broens et al., 2008a; Cohen, 1960). Taking environmental wipes to determine herd status, might therefore be a feasible option to minimize animal handling. To reduce expenses further, environmental wipes could be pooled, but this might have an effect on the performance of the test method; especially if within-herd prevalence is low (Munoz-Zanzi et al., 2006).

The objective of this study was to compare three sampling methods for MRSA classification of herds with breeding pigs. Herd classification was based on either 10 pools of 6 nasal swabs, or 5 single environmental wipes, or 1 pooled sample of 5 environmental wipes. To determine the feasibility of taking environmental wipes instead of nasal swabs for MRSA classification of herds, the number of environmental wipes required to detect MRSA in a herd at a detection limit similar to taking nasal swabs, was calculated.

MATERIALS AND METHODS

Sampling and laboratory analysis

From January to December 2008, 147 herds were randomly selected out of the national database of herds with breeding pigs. Per herd, 60 pigs were sampled using nasal swabs (Medical Wire and Equipment, MW102, United Kingdom); pigs of each age group present (sows, gilts, suckling piglets, weaned piglets and finishing pigs) were randomly sampled. Additionally, 10 moist environmental wipes (Sodibox, s1 kit ringer solution, France) were taken from surfaces in farm sections, where also pig samples were taken. These environmental wipes were taken in pairs from adjacent surfaces to enable proper comparison between results of single and pooled analysed environmental wipes.

All samples were immediately transported to the laboratory of the Dutch Animal Health Service. Samples were stored at 4 °C until processing, which occurred within 7 days after sampling. Laboratory examination took place on 10 pools of

6 nasal swabs, each pool containing swabs from only one age group and section (NASAL), 5 single environmental wipes (ENVSINGLE) and one pool of 5 environmental wipes (ENVPOOL).

Microbiological analysis was done as described in procedure 2 by Graveland et al. (2009). In brief, selective enrichment using Phenol Red Mannitol broth with 75 mg/l aztreonam and 4 mg/l ceftizoxime (PMB+; BioMérieux, NL020, France) was preceded by pre-enrichment using Mueller Hinton broth with 6.5% NaCl (MHB+). A chromogenic MRSA screen agar (Oxoid, PO5196A, United Kingdom) was used for culture and confirmation of one suspected colony per sample was done using two PCR-tests for the *S. aureus* specific DNA-fragment (Martineau et al., 1998) and the *mecA* gene (De Neeling et al., 1998) respectively.

To ensure that all samples (swabs or wipes) were totally immersed in MHB+, different volumes of MHB+ were used for each method. Pooled nasal swabs were put into 10 mL MHB+, each single environmental wipe into 100 mL MHB+ and 5 environmental wipes were pooled into 600 mL MHB+; samples were stirred and shaken by hand before and after incubation to ensure proper homogenization.

Statistical analysis

For all methods (NASAL, ENVSINGLE and ENVPOOL) a herd was classified positive if at least one sample tested positive. Pairwise comparison of methods was performed; relative sensitivity and specificity, and Cohen's kappa (Cohen, 1960) were calculated. The association between the number of positive single environmental wipes or the number of positive pools of nasal swabs per herd and the percentage of herds with an MRSA positive pool of environmental wipes was calculated using logistic regression. The probability of one (out of five) environmental wipe to be positive (= Prob) was calculated based on the number of positive pools of nasal swabs per herd; logistic regression with a random herd effect (PROC GLIMMIX; SAS, 2004) was performed. The probability to find at least 1 positive wipe (out of five) equals to $1 - (1 - \text{Prob})^5$. The number of wipes (n) needed to be e.g. 90% sure to find at least one positive environmental wipe, could then be solved from: $1 - (1 - \text{Prob})^n \geq 0.9$, yielding $n = \log(0.1)/\log(1 - \text{Prob})$.

RESULTS

Test evaluation

Apparent prevalences of MRSA positive herds ranged from 19.1% for one pool of environmental wipes (ENVPOOL), and 53.1% based on 5 single environmental wipes (ENVSINGLE) to 70.8% for 10 pooled nasal swabs (NASAL) (Table 1). The combination of NASAL and ENVSINGLE showed the highest prevalence and resulted in three extra positive herds, i.e. 72.8%. By adding ENVPOOL, no extra herds were classified positive.

The relative sensitivity was 26.9% comparing ENVPOOL with NASAL, 34.6% comparing ENVPOOL with ENVSINGLE, and 72.1% comparing ENVSINGLE with NASAL. Relative specificity was respectively 100%, 98.6% and 93.0%.

Cohen's kappa was respectively 0.18, 0.32 and 0.55 (Table 2), thus varying between very poor and moderate agreement.

Table 1. Number and apparent prevalence of MRSA positive herds based on results of three methods for MRSA classification of 147 herds with breeding pigs

Method	Type and number of samples	Number of positive herds[a]	Apparent prevalence (exact 95% CI)	
NASAL	10 pools of nasal swabs	104	70.8	(62.7-78.0)
ENVSINGLE	5 single environmental wipes	78	53.1	(44.7-61.3)
ENVPOOL	1 pool of 5 environmental wipes	28	19.1	(13.1-26.3)

[a] ≥ 1 positive sample

Table 2. Relative sensitivity, relative specificity and Cohen's kappa of three methods for MRSA classification of 147 herds with breeding pigs

Compared methods[a]	Relative sensitivity (exact 95% CI)		Relative specificity (exact 95% CI)		Cohen's kappa (95% CI)	
ENVSINGLE vs. NASAL	72.1	(62.5-80.5)	93.0	(80.9-98.5)	0.55	(0.43-0.68)
ENVPOOL vs. NASAL	26.9	(18.7-36.5)	100.0	(97.8-100.0)	0.18	(0.09-0.27)
ENVPOOL vs. ENVSINGLE	34.6	(24.2-46.2)	98.6	(92.2-100.0)	0.32	(0.21-0.43)

[a] see Table 1 for description of methods

Herd classification

On 107 farms, classified MRSA positive by either NASAL and/or ENVSINGLE, on average, 31.2% (median = 20; Q1–Q3 = 0–40) of the 5 single environmental wipes and 62.4% (median = 70; Q1–Q3 = 50–80) of the 10 pools of nasal swabs tested positive.

The percentage of herds classified positive based on ENVPOOL increased with the number of ENVSINGLE wipes per herd ($P < 0.01$), with a probability of classifying a herd MRSA positive based on the pool of environmental wipes equal to $1/(1 + \exp^{(2.65–0.7847 \times \text{number of positive single wipes per herd})})$. In 23% of the herds with only 1 out of 5 positive ENVSINGLE, the ENVPOOL tested positive. This increased to 60% in herds with 5 positive ENVSINGLE.

The percentage of herds classified positive based on ENVPOOL increased with the number of positive NASAL pools ($P < 0.01$; Fig. 1). All herds without positive NASAL pools ($n = 43$) had a negative ENVPOOL. In 6.1% (2/33) of the herds with 1–5 positive NASAL pools, the ENVPOOL tested positive as well. This increased to 64% in herds with 10 positive NASAL pools.

The percentage of herds classified positive based on ENVSINGLE increased with the number of positive NASAL pools ($P < 0.01$; Fig. 2). In 7.0% (3/43) of the herds without positive NASAL pools, one ENVSINGLE wipe tested positive. In the 5 herds with only 1 positive NASAL pool, the 5 ENVSINGLE tested negative. In 50% (5/10) of the herds with 5 positive NASAL pools, at least one ENVSINGLE tested positive. This increased to 100% (22/22) in herds with 9 or 10 positive NASAL pools.

To classify herds based on single environmental wipes only, the number of environmental wipes required to make sure that at least one positive wipe is found, was calculated. Fig. 3 shows the logistic regression lines of the probability of a wipe to be positive in relation to the number of positive pools of nasal swabs per herd. The variance explained by the random herd effect is negligible, resulting in almost similar regression lines (Fig. 3). This allowed us to do the calculation of needed wipes straight forward, since probabilities did not vary over farms (Engel et al., 1995). The probability of a single environmental wipe to be positive (= Prob) then equals $1/(1 + \exp^{(3.5527–0.4004 \times \text{number of positive pools of nasal swabs per herd})})$. Fig. 4 shows the probability of finding at least 1 positive environmental wipe out of respectively 1, 5, 10, 25 and 55 single environmental wipes; e.g. in herds with < 3 positive pools of nasal swabs, 25 single environmental wipes are required to be 90% sure that MRSA is detected. If the number of positive pools of nasal swabs increases to 6 out of 10, as in an average positive herd in this study, then 10 wipes will be sufficient to find at least one positive with a threshold of 0.9.

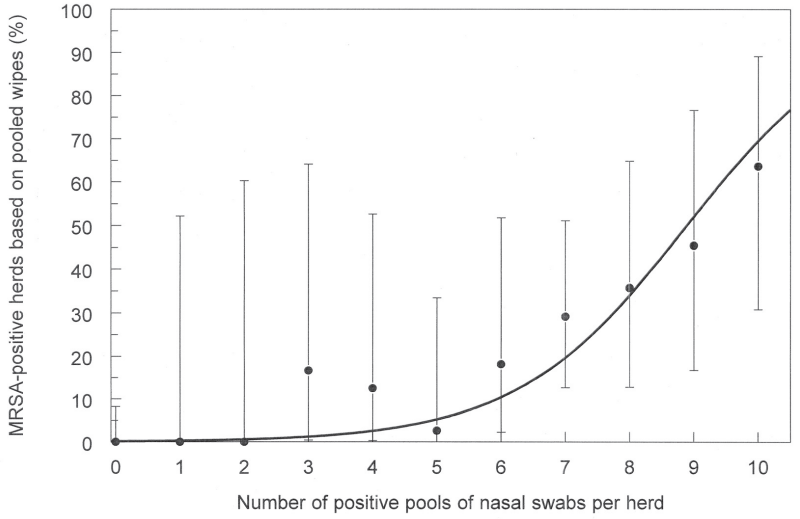

Figure 1. The percentage of MRSA-positive herds based on one pool of 5 environmental wipes related to the number of positive pools of nasal swabs per herd. The bullets represent the percentage of MRSA-positive herds per number of positive pools of nasal swabs and their exact 95%CI. The logistic regression line represents the estimated probability of a herd to be MRSA-positive given the number of positive nasal swabs: $1/(1+\exp^{(6.6067-0.743 \times \text{number of positive pools of nasal swabs per herd})})$.

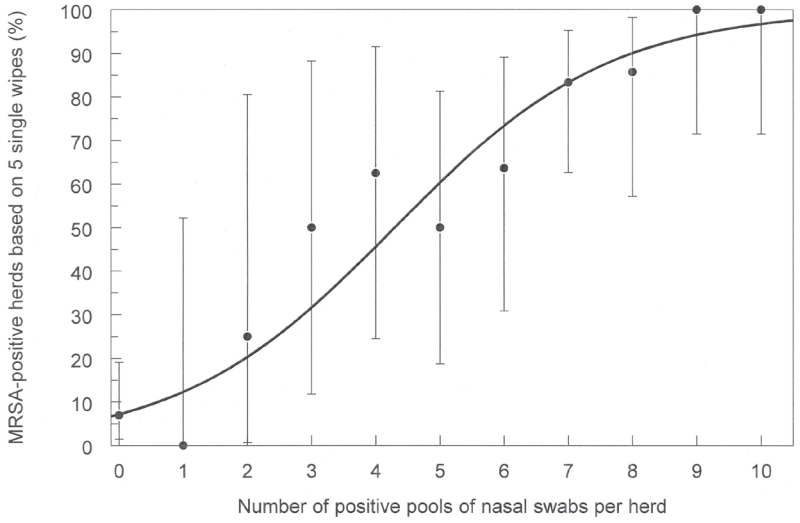

Figure 2. The percentage of MRSA-positive herds based on 5 single environmental wipes related to the number of positive pools of nasal swabs per herd. The bullets represent the percentage of MRSA-positive herds per number of positive pools of nasal swabs and their exact 95%CI. The logistic regression line represents the estimated probability of a herd to be MRSA-positive given the number of positive nasal swabs: $1/(1+\exp^{(2.5554-0.5947 \times \text{number of positive pools of nasal swabs per herd})})$.

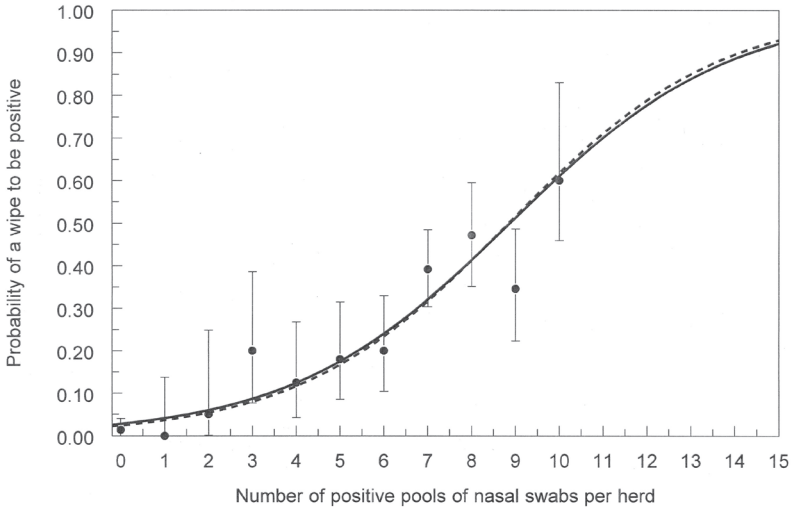

Figure 3. The estimated probability of at least one wipe to be positive related to the number of positive pools of nasal swabs per herd based on logistic regression with (---) and without (———) random herd effect. The probability without random effect: $1/(1+\exp^{(3.5527-0.4004 \times \text{number of positive pools of nasal swabs per herd})})$. The bullets represent the proportion of positive wipes per number of positive pools of nasal swabs and their exact 95%CI.

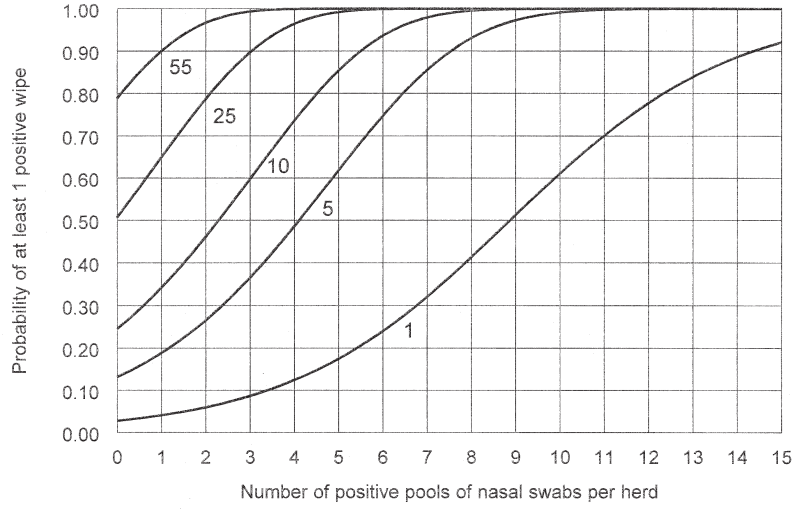

Figure 4. The estimated probability to find at least one positive wipe out of respectively 1, 5, 10, 25 and 55 wipes based on the number of positive pools of nasal swabs per herd.

DISCUSSION

Classifying herds based on a combination of 10 pools of nasal swabs and 5 single environmental wipes showed the highest apparent prevalence, i.e. 72.8%, in our study in 147 herds with breeding pigs.

With 10 pools of nasal swabs more MRSA positive herds were detected than with 5 single environment wipes. This might be due to the difference in 10 vs. 5 samples to classify a herd, but might also be due to a difference in MRSA load between pigs and the environment. Nevertheless, the agreement between herd classification based on 5 single environmental wipes and based on 10 pools of nasal swabs was moderate ($\kappa = 0.55$), which is promising for future monitoring and surveillance programs. The number of environmental wipes needed for such programs depends on several factors, e.g. required detection limits and within-herd prevalence. We found that in low prevalent herds (e.g. herds with < 3 positive pools of nasal swabs), 25 single environmental wipes are required to be 90% sure that MRSA is detected at a detection limit similar to analysing 10 pools of nasal swabs. As we obtained the required sample size by extrapolation outside observed data, these sample sizes should be regarded as guidelines, and validation should be performed in field studies.

The agreement between herd classification based on one pool of 5 environmental wipes and 5 single environmental wipes was poor ($\kappa = 0.32$). A possible explanation might be that the wipes are not taken from exactly the same surfaces. Despite our effort to take the wipes in pairs from adjacent surfaces, this is no guarantee for similar MRSA load on wipes. This seems an unsatisfactory explanation, as only in one herd with a positive pool of environmental wipes no positive single environmental wipes were found, whereas in 51 herds with at least one positive single environmental wipe the pool of environmental wipes was negative. Another possible explanation might be false-negative results due to a lower density of MRSA in the pooled samples. The higher volume of pre-enrichment broth used for pooled wipes will decrease the density of MRSA in the MHB^+, especially when MRSA negative wipes are present in the pool. Besides, homogenization of samples with a pool of 5 environmental wipes might have been less effective than in samples with only one single environmental wipe. An additional explanation for the poor agreement might be the higher possibility of presence of inhibitory components or competing organisms present in a pooled sample compared to a single sample. A mix of non-pathogenic bacterial flora might repress staphylococcal growth (Peterson et al., 1962), and antibiotic treated feed may contribute to the antimicrobial component of dust present in pig buildings and therefore inhibit bacterial growth as well (Murphy et al., 2007).When these organisms or components are present in the dust on one single environmental wipe, this sample, and even the entire pool, might turn out to be false-negative. Nevertheless, in the case of testing single environmental wipes, there are still four environmental wipes left used for herd classification.

To conclude, environmental wipes can be used as a relatively easy and non-invasive method to classify herds for MRSA, but the number required

depends on the within-herd prevalence. Environmental wipes must be examined individually, since pooling resulted in a large reduction of the number of detected positive herds. Consequently, the apparent herd prevalence is much lower based on pooled environmental wipes than based on single environmental wipes and/or pools of nasal swabs. Prevalence surveys based on pooled environmental wipes therefore result in substantial underestimation of the true prevalence, especially when within-herd prevalences are low, and, with that, quantification of risk factors based on these results would be questionable.

Acknowledgements

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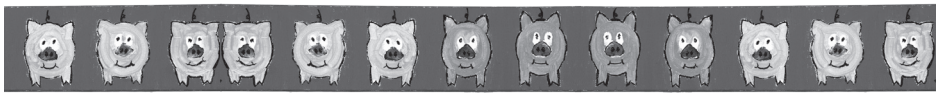
CHAPTER 2.2

MRSA CC398 IN THE PIG PRODUCTION CHAIN



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KEYWORDS

- *Staphylococcus aureus*
- MRSA
- Pigs
- Transmission

ABSTRACT

In 2005, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA CC398) was found in pigs and people in contact with pigs. The structure of the pig production chain in high technology pig husbandry enables pathogens to spread during animal trading, with an increasing prevalence in herds further down the chain. The objective of this study was to quantify the effect of the MRSA status of the supplying herd on the MRSA status of the receiving herd in order to gain more insight into the role of animal trading as a transmission route for MRSA CC398. Nasal samples (60–80 pigs per herd) were collected from 38 herds; in 20 herds, environmental samples were collected as well. Ten MRSA positive herds (based on the results of nasal swabs of 10 individual pigs per herd) from a prior study were included in the data analysis. Herds were classified as MRSA positive if at least one sample tested positive. The 48 herds were part of 14 complete (40 herds) and 4 incomplete (8 herds) pig production chains. Fifty-six percent of the herds were classified as MRSA positive. MRSA positive herds were observed at the start (breeding herds), middle (farrowing herds) and the end (finishing herds) of the pig production chain. All of the herds in 8 chains tested MRSA positive, all of the herds in 5 chains tested MRSA negative and in the remaining 5 chains, MRSA positive and MRSA negative herds were detected. Seven *spa* types were found, which were all previously confirmed to belong to CC398. All of the isolates were susceptible to mupirocin, linezolid, rifampicin, fusidic acid and cotrimoxazole. Resistance against tetracycline, erythromycin and clindamycin was found in 100, 74 and 76% of the isolates, respectively. Seventy-nine percent of herds with a MRSA positive supplier of pigs were MRSA positive, whereas 23% of herds with a MRSA negative supplier were MRSA positive (OR = 10.8; 95% CI: 1.5–110.1; $P = 0.011$). The presence of entirely MRSA positive and MRSA negative chains and the strong association between the MRSA status of herds and their suppliers illustrates a large risk associated with purchasing pigs from MRSA positive herds; a top-down strategy for future control programs is, therefore, a basic requirement. However, 23% of herds with a MRSA negative supplier were MRSA positive and furthermore, 46% of the herds at the top of the pig production chain without a supplier tested MRSA positive. This underlined the need for the identification of additional risk factors for MRSA.

INTRODUCTION

In 2005, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA CC398) was found in pigs and people in contact with pigs in The Netherlands (Voss et al., 2005). Since then, several other countries have detected MRSA CC398 in pig herds and other livestock (Graveland et al., 2008; Khanna et al., 2008; EFSA, 2009; Kock et al., 2009; Smith et al., 2009; Van Den Broek et al., 2009; Mulders et al., 2010). The prevalence of MRSA positive pig herds varied from 23 to 70%, which might have been due to inconsistent sampling and laboratory techniques. Another explanation might be the selection of herds because risk factors for the introduction and persistence of MRSA on breeding herds might be completely different from risk factors for finishing herds. In the studies mentioned above, no explicit distinction was made between MRSA prevalence in different herd types, and reports on risk factors are limited. Van Duijkeren et al. (2008) showed in a pilot study, that 5 out of 6 herds supplying pigs to MRSA positive herds were MRSA positive, which indicated transmission of MRSA within the pig production chain by the purchase of pigs. As breeding pigs constitute the top of the pig production chain, MRSA CC398 has the possibility to spread to a large number of farrowing herds by the trade of gilts and subsequently from these farrowing herds to an even larger number of finishing herds by the trade of piglets. The study objective was to quantify the effect of the MRSA status of the supplying herd on the MRSA status of the receiving herd to gain insight into the role of animal trade as a transmission route for MRSA CC398.

MATERIALS AND METHODS

Study design and sampling

In 2007 and 2008, 38 herds were sampled, including 11 breeding, 5 breeding-to-farrowing, 7 farrowing, 5 farrowing-to-finishing, and 10 finishing herds. Breeding herds were defined as herds with (pure) breeding pigs that supply gilts and/or boars to farrowing herds; no pigs enter these herds. Farrowing herds were defined as herds with farrowing pigs that supply pigs to finishing herds. Finishing herds were defined as herds that supply pigs for slaughter. Herds sometimes combined 2 of the previously mentioned disciplines, i.e., breeding and farrowing or farrowing and finishing. A pig production chain was defined as a number of pig farms linked to each other by the trade of animals. To enable reliable quantification of the effect of the MRSA status of the supplying herd on the MRSA status of the receiving herd, only herds with a maximum of 2 pig suppliers were selected. Herds within the same pig production chain were identified using the Dutch Identification and Registration System. Registration data were checked to make sure that farms did not change pig suppliers within a year before sampling. The time between sampling herds within a chain varied from several hours to 6 months. Nasal swabs (Medical Wire and Equipment, MW102, United Kingdom) were collected from either 60 or 80 pigs per herd. This sample size enabled MRSA to be detected in herds with a within-herd prevalence of 2–5%. Swabs were taken

from each age group (sows, suckling piglets, weaners, finishers and rearing pigs) present in the herd. Additional samples, i.e., 5 environmental wipes (Sodibox, s1 kit ringer solution, France) were collected in 20 herds. Information on batch treatment with antimicrobials was available for 34 farms that participated in another study.

Data from 10 herds (2 breeding, 1 breeding-to-farrowing, 4 farrowing and 3 finishing herds) which were used by Van Duijkeren et al. (2008) in a previous study, were included in the analysis. In that previous study, 10 individual pigs per herd were sampled to determine the MRSA status. Ten samples per herd enabled MRSA to be detected in herds with a within-herd prevalence of 25%. Therefore, only MRSA positive herds found it that study were included in our study.

The 48 herds were part of 14 complete (40 herds) and 4 incomplete (8 herds) independent pig production chains. Only 1 herd was missing in each incomplete chain due to noncooperative farmers. Pig production chains consisted of at least 2 and at most 5 herds; two herds were used in the case of incomplete chains or when herds combining 2 disciplines were involved.

Laboratory analysis

All samples were sent to the Animal Health Service for analysis, which took place within 10 days after sampling. Analyses were performed on individual environmental wipes and pooled nasal swabs (4–6 swabs per pool) with each pool containing swabs from just one section of the herd and age group. First, samples were enriched using Mueller Hinton Broth with 6.5% NaCl (MHB+). Nasal swabs were placed into 10 mL MHB+ and environmental wipes were placed into 100 mL MHB+. After 18 h of aerobic incubation at 37 °C, 1 mL of MHB+ was transferred into 9 mL of Phenol Red Mannitol Broth with 75 mg/L aztreonam and 4 mg/L ceftizoxime (PMB+; BioMérieux, NL020, France). This selective enrichment broth was incubated aerobically for 18 h at 37 °C. A loop-full of PMB+ was spread onto sheep blood agar (Oxoid, PB5008A, United Kingdom) and a chromogenic MRSA screen agar (Oxoid, PO5196A, United Kingdom). One suspected colony per sample was confirmed by 2 PCR tests for the *S. aureus* specific DNA-fragment (Martineau et al., 1998) and the *mecA* gene (De Neeling et al., 1998). To confirm that MRSA isolates belonged to CC398 and to gain insight into the relatedness of isolates within a chain, isolates were typed by *spa* typing (Harmsen et al., 2003). Antimicrobial susceptibilities of at least 1 isolate per herd were determined quantitatively by broth microdilution with cation-adjusted Mueller Hinton broth according to ISO standard 20776-1:2006. For broth microdilution, microtitre trays were used with custom-made panels of dehydrated dilution ranges of antibiotics (Sensititre®, Trek Diagnostic Systems, Basingstoke, UK). The ATCC strains *Enterococcus faecalis* 29212 and *Staphylococcus aureus* ATCC 29213 were included for quality control. The minimum inhibitory concentrations were defined as the lowest concentrations without visible growth. Breakpoints for classification of resistance and susceptibility were determined based on international and national standards (Table 1; www.eucast.org; CLSI, 2007). Human clinical breakpoints were primarily used to investigate resistance against antimicrobials used in human medicine.

The antimicrobials tested in this study were amikacin, ciprofloxacin, clindamycin, cotrimoxazole, erythromycin, fusidic acid, gentamicin, linezolid, mupirocin, neomycin, rifampicin and tetracycline.

Table 1. Breakpoints used for classification of resistance and susceptibility for different antimicrobials and the international standard in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Antimicrobial	Breakpoint	International standard
Amikacin	R ≥ 32	EUCAST
Ciprofloxacin	R > 1	EUCAST
Clindamycin	R ≥ 4	CLSI M100-S17
Cotrimoxazole	R ≥ 4	CLSI M100-S17
Erythromycin	R ≥ 8	CLSI M100-S17
Fusidic acid	R ≥ 8	CRG
Gentamicin	R > 1	EUCAST
Linezolid	R > 4	EUCAST
Mupirocin	R > 4	not available
Neomycin	R ≥ 8	not available
Rifampicin	R ≥ 4	CLSI M100-S17
Tetracycline	R ≥ 32	CLSI M100-S17

EUCAST = European Committee on Antimicrobial Susceptibility Testing;
CRG = Dutch Committee on guidelines on Antimicrobial Susceptibility Testing;
CLSI = Clinical and Laboratory Standards Institute.

Data analysis

Herds were classified as MRSA positive if at least one sample (individual nasal sample, pool of nasal samples or environmental sample) tested positive. The association between the MRSA status of the receiving herds and the MRSA status of their supplier and application of batch treatments with antimicrobials were calculated using exact logistic regression analysis (SAS, 2004). The strength of association is presented in terms of odds ratios (OR). The estimated attributable fraction was calculated using the following equation: (OR-1)/OR (Noordhuizen et al., 2001).

Cohen's kappa, which is a measure of agreement between diagnostic methods, was calculated to compare the microbiological results of pooled animal samples and environmental samples (Cohen, 1960).

RESULTS

Fifty-six percent (27/48; 95% CI: 41–71) of the herds were classified as MRSA positive. MRSA positive herds were observed in all types of herds within the pig production chain, ranging from 20.0% MRSA positive farrowing-to-finishing herds (1 out of 5) to 100.0% MRSA positive breeding-to-farrowing herds (6 out of 6) (Table 2). On average, 42% of the individual pigs tested MRSA positive in herds classified positive based on individual pig samples (n = 10). On average, 50% of the pooled samples and 20% of the environmental samples were MRSA positive in herds classified as positive based on pooled samples (n = 7) or on pooled and environmental samples (n = 10). In 3 herds, environmental wipes tested MRSA negative, even though the herd was classified as MRSA positive based on at least one MRSA positive pool of nasal swabs. In the remaining 17 herds in which environmental samples were taken, the results of the environmental and pooled samples were consistent with the MRSA status of the herd; in 7 herds, environmental and pooled samples tested MRSA positive and in 10 herds, all of the samples tested MRSA negative. Cohen's kappa, a measure of agreement between the MRSA classification of a herd based on environmental wipes and pools of nasal swabs, was 0.70 (95% CI: 0.40–1.00).

All of the herds tested MRSA positive in 8 pig production chains, including 3 incomplete chains. All herds tested MRSA negative in 5 chains. MRSA positive and MRSA negative herds were observed in the remaining 5 chains, including 1 incomplete chain (Table 3). Sampling within a chain took place within 1 month in 11 chains. In the other 7 chains, the time intervals between sampling moments were between 1 and 6 months.

In 27 out of the 48 herds, the MRSA status of the supplier was known. The other 21 herds were breeding (n = 13) or breeding-to-farrowing (n = 6) herds without supplier or herds of which the supplier was not sampled (n = 2). Seventy-nine percent of the herds with a MRSA positive supplier were MRSA positive, whereas 23% of herds with a MRSA negative supplier were MRSA positive (OR = 10.8; 95% CI: 1.5–110.1; P = 0.011) (Table 4). The estimated attributable fraction was 0.91, which indicated that the MRSA status of 91% of the MRSA positive herds was attributed to the MRSA positive status of their supplier.

Antimicrobials were used as batch treatments on 74% (25/34) of the herds in which information on antimicrobial use was available. Herds that used antimicrobials as batch treatments were MRSA positive more often (76%) than herds that used antimicrobials incidentally (22%; OR = 10.2; 95% CI: 1.4–126.1; P = 0.015) (Table 4). Bivariable logistic regression with supplier status and batch treatment was possible for only 21 herds and showed no significant effect of batch treatments on MRSA status. Nine out of 14 herds (64%) that used batch treatments were MRSA positive, while only 2 out of 7 herds (30%) that did not use batch treatments were MRSA positive (OR = 2.3; 95% CI: 0.1–48.0; P = 0.836). The effect of supplier status in the bivariable analysis (OR = 10.2; 95% CI: 1.1–156.8; P = 0.042) was consistent with the effect estimated in the univariable analysis on these 21 herds.

All of the MRSA isolates (*n* = 154) were spa typed (Table 3 and 5). Seven *spa* types were identified, which were closely related and formerly confirmed to belong to CC398. *Spa* types t011 and t108 were detected most frequently and were identified in 42% and 28% of the isolates and in 48% and 52% of the positive herds, respectively. In 5 out of 27 positive herds, t011 and t108 occurred simultaneously within the herd. The less frequently identified *spa* types t567 (8%), t899 (6%), t943 (13%), t1939 (1%) and t2503 (1%), were found in herds within 1 pig production chain. *Spa* type t899 together with t1939 in chain M and spa type t943, t2503, t011 and t108 were found in chain F. *Spa* type t567 was only found in chain O. In 20 out of 27 MRSA positive herds, only 1 *spa* type was found. In 6 herds, 2 different *spa* types were found, and in 1 herd, 4 different *spa* types were found (Table 3). In all of the cases where 2 *spa* types were found in 1 herd, there were 2 isolates that differed by only 1 repeat in the alignment of tandem repeats in the staphylococcal protein A region (Table 5).

Antimicrobial susceptibilities were determined for 86 MRSA isolates (Table 6). All of the isolates were susceptible to mupirocin, linezolid, rifampicin, fusidic acid and cotrimoxazole. Resistance against tetracycline, erythromycin and clindamycin was found frequently (100.0%, 74.4% and 75.6% of the isolates, respectively) and often in combination. Combined resistance against erythromycin and clindamycin was found in 67.4% (58/86) of the isolates. Resistance against aminoglycosides ranged from 3.5% for amikacin to 15.9% for neomycin. Resistance against ciprofloxacin was found in 2 isolates, *spa* types t011 and t108 in chain P. Multiresistance (resistance against > 3 different antimicrobial classes) was found in 9.3% (8/86) of the isolates: 6 times in *spa* type t011 and 2 times in *spa* type t108.

Table 2. Total number of herds and number and percentage with exact 95% confidence interval of MRSA positive herds in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Herd type	*n* Total	*n* Positive	% Positive	95% CI
Breeding	13	6	46.2	19.2 - 74.9
Breeding-to-farrowing	6	6	100.0	54.1 - 100.0
Farrowing	11	5	45.5	16.8 - 76.6
Farrowing-to-finishing	5	1	20.0	0.5 - 71.6
Finishing	13	9	69.2	38.6 - 90.9
Total	48	27	56.3	41.2 - 70.5

Table 3. Total number and number of positive herds, samples per herd, herd type, pig supplier, MRSA status, *spa* type, resistance pattern and application of batch treatments in the pig production chains in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Chain	Herd	Herd type	Pig supplier	MRSA status	Positive (total) number of pools	wipes	pigs	*Spa* type *(n)*	Resistance pattern *(n)*	Batch treatment
Negative chains										
A [a]	1	a	-	neg	0 (20)					-
	2	c	1	neg	0 (15)					-
	3	e	2	neg	0 (10)	0 (5)				no
B [a]	4	a	-	neg	0 (20)					-
	5	a	-	neg	0 (20)					-
	6	d	4 / 5	neg	0 (10)	0 (5)				yes
C [a]	7	a	-	neg	0 (20)					-
	8	c	7	neg	0 (15)					-
	9	e	8	neg	0 (10)	0 (5)				no
D [a]	10	a	-	neg	0 (20)					-
	11	d	10	neg	0 (10)	0 (5)				yes
E	12	a	-	neg	0 (15)					no
	13	d	12	neg	0 (20)					yes
	14	d	12	neg	0 (20)					no
'Mixed' chains										
F	15	a	-	pos	1 (20)			t108 (1)	CIAENT (1)	-
	16	c	15	neg	0 (15)					-
	17	b	-	pos	14 (15)			t943 (13)	CIET (13)	-
								t2503 (1)	CIET (1)	
	18	e	16 / 17	pos	10 (10)			t108 (1)	CIET (1)	-
								t011 (1)	CIENT (1)	
								t943 (7)	CIET (7)	
								t2503 (1)	CIET (1)	
G [a]	19	a	-	pos	2 (20)			t108 (2)	CIET (2)	-
	20	c	19	neg	0 (10)	0 (5)				yes
	21	e	20	pos	2 (10)			t011 (2)	AGT (1)	no
									GT (1)	
H [a,b]	22	c	n.k.	neg	0 (10)	0 (5)				yes
	23	e	22	pos	1 (10)			t011 (1)	T (1)	-
I	24	a	-	pos	6 (10)	1 (5)		t011 (7)	GT (1)	yes
	25	c	24	pos	5 (10)	0 (5)		t108 (5)	ET (1)	yes
	26	e	25	neg	0 (10)	0 (5)				no

J	27	a	-	neg	0 (10)	0 (5)				no
	28	d	27	pos	5 (10)	1 (5)		t108 (6)	GT (1)	yes
	29	c	27	neg	0 (10)	0 (5)				yes
	30	e	29	neg	0 (10)	0 (5)				no
Positive chains										
K [a]	31	a	-	pos	4 (20)			t011 (3)	T (1)	yes
									CIEGNT (2)	
								t108 (1)	CIET (1)	
	32	c	31	pos			1 (10)	t108 (1)	CIET (1)	no
	33	e	32	pos			3 (10)	t108 (3)	CIET (3)	yes
L	34	b	-	pos			8 (10)	t108 (8)	CIET (8)	yes
	35	e	34	pos			2 (10)	t011 (1)	CIET (1)	yes
								t108 (1)	CIET (1)	
M [b]	36	c	n.k.	pos			8 (10)	t899 (7)	CIET (7)	yes
								t1939 (1)	CIET(1)	
	37	e	36	pos			2 (10)	t899 (1)	T (2)	yes
N [b]	38	a	-	pos			2 (10)	t108 (2)	ET (2)	yes
	39	c	38	pos			3 (10)	t108 (3)	ET (3)	yes
O [b]	40	a	-	pos			4 (10)	t567 (4)	T (2)	yes
									CIT (2)	
	41	c	40	pos			9 (10)	t567 (9)	CIT (5)	yes
									T (4)	
P	42	b	-	pos	9 (10)	1 (5)		t011 (6)	CiCIET (1)	yes
								t108 (4)		
	43	e	42	pos	6 (10)	2 (5)		t108 (7)	CiCIET (1)	yes
								t011 (1)		
Q	44	b	-	pos	7 (10)	1 (5)		t011 (8)	ACIEGT (1)	yes
	45	b	-	pos	6 (10)	0 (5)		t011 (6)	CIET (1)	yes
	46	e	44 / 45	pos	10 (10)	3 (5)		t011 (13)	CIEGNT (1)	yes
R	47	b	-	pos	5 (10)	0 (5)		t011 (5)	NT (1)	yes
	48	e	47	pos	9 (10)	1 (5)		t011 (10)	CIENT (1)	-

[a] Time between sampling 1-6 months; n.k. = not known

[b] Incomplete chain (1 herd is missing)

a = breeding herd; b = breeding-to-farrowing herd; c = farrowing herd; d = farrowing-to-finishing herd; e = finishing herd

A = amikacin, Ci = ciprofloxacin, Cl = clindamycin, E = erythromycin, G = gentamicin, N=neomycin, T = tetracycline

Table 4. Frequency (*n* and %), MRSA prevalence (%) and odds ratio (OR) for a herd to be MRSA positive with 95% confidence interval (CI), and exact *P*-value, resulting from univariable analysis for status of supplier and antimicrobial batch treatment in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Variable	Category	Frequency	MRSA (%)	OR	95% CI	*P*-exact
Status supplier	Positive	14 (51.9)	78.6	10.8	1.5-110.1	0.011
	Negative	13 (48.2)	23.1	Ref.		
Batch treatment	Yes	25 (73.5)	76.0	10.2	1.4-126.1	0.015
	No	9 (26.5)	22.2	Ref.		

Table 5. *Spa* types and their repeat succession of 154 MRSA isolates: total number and percentage of all isolates, number and percentage of positive herds (out of 27 positive herds) and number and percentage of positive chains (out of 13 positive chains) in which the type was found in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Spa type	Repeat succession[a]	Isolates		Positive herds		Positive chains	
		n	%	*n*	%	*n*	%
t011	08-16-02-25-34-24-25	64	41.6	13	48.1	9	69.2
t108	08-16-02-25-24-25	45	29.2	14	51.9	8	61.5
t567	08-02-25-24-25	13	8.4	2	7.4	1	7.7
t899	07-16-23-02-34	9	5.8	2	7.4	1	7.7
t943	08-16-02-25-25-24-25	20	13.0	2	7.4	1	7.7
t2503	08-16-02-25-25-25-24-25	2	1.3	2	7.4	1	7.7
t1939	07-23-02-34	1	0.6	1	3.7	1	7.7

[a] www.spaserver.ridom.de

Table 6. Number of isolates tested per *spa* type with the resistance percentage for 12 antimicrobials in a study on MRSA CC398 in the pig production chain (The Netherlands, 2007/2008).

Spa type /	t011		t108		t567		t899		t943		t1939		t2503		Total	
Antimicrobial	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Amikacin	15	13	26	0	13	0	9	0	20	0	1	0	2	0	86	4
Ciprofloxacin	15	7	26	4	13	0	9	0	20	0	1	0	2	0	86	2
Clindamycin	15	60	26	73	13	54	9	78	20	100	1	100	2	100	86	76
Cotrimoxazole	15	0	26	0	13	0	9	0	20	0	1	0	2	0	86	0
Erythromycin	15	60	26	96	13	0	9	78	20	100	1	100	2	100	86	74
Fusidic acid	14	0	8	0	-	-	-	-	20	0	-	-	2	0	44	0
Gentamicin	15	47	26	0	13	0	9	0	20	0	1	0	2	0	86	9
Linezolid	14	0	8	0	-	-	-	-	20	0	-	-	2	0	44	0
Mupirocin	14	0	8	0	-	-	-	-	20	0	-	-	2	0	44	0
Neomycin	14	43	8	13	-	-	-	-	20	0	-	-	2	0	44	16
Rifampicin	14	0	8	0	-	-	-	-	20	0	-	-	2	0	44	0
Tetracycline	15	100	26	100	13	100	9	100	20	100	1	100	2	100	86	100

DISCUSSION

This study observed completely MRSA negative and positive pig production chains and an 11 times higher odds for herds with a MRSA positive supplier to be MRSA positive. These results confirm the hypothesis of Van Duijkeren et al. (2008) that animal trading was an important factor in the transmission of MRSA between pig herds. The occurrence of the same *spa* type, including some uncommon *spa* types and ciprofloxacin resistance within the same production chain supports the likelihood of MRSA transmission between pig herds by the trade of animals. However, the sample size was too small to statistically validate the findings on *spa* types and resistance patterns. The MRSA status of a vast majority of MRSA positive herds was attributed to the MRSA positive status of their supplier. However, this was estimated by univariable analysis, which assumes that the relationship is causal, and confounding is absent.

The 2 predominant *spa* types, t011 and t108, observed in this study have been found in high numbers in previous Dutch studies (De Neeling et al., 2007; Van Duijkeren et al., 2008; Huijsdens et al., 2009). These 2 *spa* types are closely related and differ by only one repeat in the alignment of tandem repeats in the staphylococcal protein A region, which was similar to two other combinations that were found in our study (*spa* types t943 and t2503 and *spa* types t899 and t1939). Little is known about the incidence of mutations and, subsequently, conversion from one *spa* type to another. Therefore, the simultaneous occurrence of two closely related *spa* types within one chain or even one herd could be a result of (1) two independent introductions of MRSA or (2) one single introduction followed by a mutation. The finding in our study of three combinations of *spa* types differing by just one repeat, indicated that new *spa* types might have emerged through mutation, or more specifically, by a loss of repeats. As the staphylococcal protein A is just a small part of the whole organism, other typing techniques could be useful in investigating the relatedness of different *spa* types. Experiments in vitro and in vivo might help to estimate the incidence of mutations in the staphylococcal protein A region.

The good agreement observed between the classification of herds based on results of the pools of nasal swabs and the results of the environmental samples were consistent with a previous study (Broens et al., 2011a). The detection of MRSA in environmental samples implied the possibility of indirect transmission of MRSA between pigs within a herd and between pigs and humans (Van Den Broek et al., 2009).

The antimicrobial resistance patterns found were similar to other studies on MRSA CC398 (De Neeling et al., 2007; Smith et al., 2009; Wagenaar and Van De Giessen, 2009). High prevalence of resistance against macrolides, lincosamides and tetracyclins has been observed in previous studies. Resistance against tetracycline, lincosamides and macrolides in MRSA CC398 appears to depend on the presence of the *tetM*-gene (tetracycline) and the *erm*-genes (lincosamides and macrolides) (Witte et al., 2007). Resistance against ciprofloxacin appears to be rare in pig isolates, in contrast to poultry and veal isolates (Wagenaar and Van De

Giessen, 2009; Mulders et al., 2010). In Dutch poultry and veal husbandry, batch treatment with quinolones is frequently applied, whereas in Dutch pigs, the use of quinolones is uncommon (MARAN, 2008). The use of quinolones was not reported in either of the herds in which a ciprofloxacin-resistant isolate was identified. Resistance against mupirocin, linezolid, rifampicin and fusidic acid was not identified, which is important because these antimicrobials are considered important to the control of MRSA in human medicine.

The presence of completely MRSA negative chains and the strong association between the MRSA status of herds and their suppliers suggested that a top-down strategy should be a prerequisite for future control programs. Such programs are based on the principle of top-down eradication, which ensures the absence of the 'disease' from the entire pig production chain as described for *Salmonella* in broiler chickens in Denmark (Wegener et al., 2003). However, 46% of the self-supplying herds and 23% of the herds with a MRSA negative supplier tested MRSA positive. Therefore, more research is needed to elucidate additional risk factors for the introduction and persistence of MRSA in pig herds.

'Mixed' chains were observed in addition to completely positive or negative chains. In some cases, the period between sampling of the herds within one chain was very long (up to 6 months), which might explain some of the 'mixed' chains. The MRSA status of the herd could have changed within this period. In addition, there were chains in which information on one herd was missing, because the farmer was not willing to cooperate.

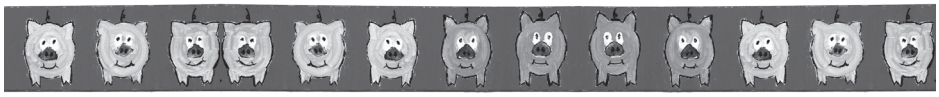
Batch treatments with antimicrobials resulted in a higher prevalence in herds that were subjected to batch treatments compared herds that were not subjected to batch treatments. However, in multivariable analysis on a smaller number of herds, due to incomplete information, the effect of batch treatment was smaller and not significant. A multivariable analysis on a large number of herds is needed to identify and quantify the effect of antimicrobial use on MRSA.

CONCLUSION

The results of this study illustrated that the MRSA status of a pig supplier highly affects the MRSA status of the receiving herd. A top-down control strategy for MRSA is therefore a basic requirement in the pig production chain. However, additional risk factors for MRSA need to be identified because not all MRSA positive herds could be attributed to pigs received from MRSA positive herds.

CHAPTER 2.3

PREVALENCE AND RISK FACTOR ANALYSIS OF LIVESTOCK-ASSOCIATED MRSA POSITIVE PIG HERDS IN THE NETHERLANDS



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KEYWORDS

- Methicillin resistant *Staphylococcus aureus* (MRSA)
- Risk factors
- Prevalence
- Pigs

ABSTRACT

In 2005, methicillin resistant *Staphylococcus aureus* was found in pig herds and in humans in contact with pigs. To determine the prevalence of, this now-called livestock-associated (LA-) MRSA among pig herds in the Netherlands and to identify and quantify risk factors, an observational study of 202 pig herds was performed between 2007-2008. Five environmental wipes and 60 nasal swabs from each herd were collected, and microbiological analysis was performed on single environmental samples and pooled nasal samples. A herd was considered MRSA positive if ≥ 1 sample tested positive. The prevalence of MRSA positive herds was 67% in breeding herds and 71% in finishing herds. Multivariable logistic regression analysis was then performed on data from 171 breeding herds. The number of MRSA positive herds increased from ~30% at the start to ~75% at the end of the study, most likely due to transmission between herds. The prevalence of MRSA increased with herd size, as ~40% of smaller herds (< 250 sows) were MRSA positive compared to > 80% of larger herds (> 500 sows). Other risk factors (e.g. antimicrobial use, purchase of gilts and hygiene measures) were not significantly associated with MRSA, though associated with herd size. Herd size appeared to be a compilation of several factors, which made larger herds more often MRSA positive.

INTRODUCTION

In 2005, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA) was found in pig herds and in humans in contact with pigs in the Netherlands (Voss et al., 2005) and studies have subsequently detected this, so-called livestock-associated (LA) clone in pigs in other countries as well as in other livestock (Broens et al., 2008a; EFSA, 2009; Smith et al., 2009; Graveland et al., 2010; Mulders et al., 2010).

The reported prevalence of LA-MRSA among pig herds greatly varied, ranging from 0% in several European Union (EU) member states to 81% in a Dutch study performed at pig slaughterhouses (De Neeling et al., 2007; EFSA, 2009). This range can be due to differences in the number of pigs analysed, sample type and size, microbiological procedures and transmission between batches from different herds during animal trading (Noordhuizen et al., 2001; Graveland et al., 2009; Broens et al., 2010, 2011a). Risk factors for humans living or working on pig and veal farms have been identified (Van Den Broek et al., 2009; Graveland et al., 2010) and an EU-wide baseline survey revealed preliminary factors associated with MRSA contamination of breeding holdings (EFSA, 2010). Risk factors for MRSA positive pig herds appear to be herd size, purchase of pigs and antimicrobial use (Van Duijkeren et al., 2008; EFSA, 2010; Broens et al., 2011c).

In this study, we aimed to determine the prevalence of MRSA positive pig herds as well as to identify and quantify risk factors for positive breeding herds.

MATERIAL AND METHODS

Study design and sampling

The study was conducted from January 2007 to December 2008. Nasal swabs (MW102, Medical Wire and Equipment) were collected from pigs from each age group (sows, suckling piglets, weanling piglets, replacement pigs and finishing pigs) present in a herd, where a herd is defined as a pig holding at one location owned by one owner. Swabs from 6 pigs per age group were combined into a pooled sample up to a total of 10 pooled samples (i.e. 60 nasal swabs) per herd. In addition, 5 environmental wipes (s1 kit ringer solution, Sodibox) were collected from horizontal surfaces, e.g. top of fences, steel bars and ventilators, from 5 different animal sections (1 wipe per sampled age group).

To identify risk factors, questionnaires were used to gather information on herd characteristics (Table 1).

Coordination and microbiological analyses were performed at the National Institute for Public Health and the Environment (Lab I) in 2007, and all samples were collected according to protocol by employees of the Dutch Food and Consumer Product Safety Authority. A total of 50 pig herds were randomly selected, resulting in 31 finishing herds and 19 breeding herds in this year. Coordination, sample collection and microbiological analyses were transferred to the Animal Health Service (Lab II) in 2008. We then focused on breeding herds, as: (1) preliminary results from a vertical transmission study showed that the MRSA

Table 1. Herd characteristics (in categories) derived from the questionnaire, with the number of herds, herd prevalence based on 171 Dutch pig herds, and pool prevalence based on 1699 pooled samples.

Variable	Freq (*n* herds)	Herd prevalence (%)	Pool prevalence (%)
Period (2007-1; 2007-2; 2008-1; 2008-2) [a]	16; 4; 91; 60	37.5; 25.0; 70.3; 73.3	25.6; 25.0; 44.9; 46.3
Season (spring; summer; autumn; winter) [a]	71; 42; 32; 26	73.2; 64.3; 75.0; 46.2	48.9; 45.5; 43.4; 23.6
Pig density region (≤ 17; > 17 pigs/ha) [a]	88; 83	59.1; 75.9	40.2; 46.2
Herd category (A;B) [a]	80; 91	75.0; 60.4	51.3; 35.9
Herd size (<250; 250-600; > 600) [a]	59; 79; 33	49.2; 72.2; 87.9	24.7; 48.0; 63.9
Presence of finishing pigs (<100; > 100) [a]	108; 63	70.4; 61.9	44.7; 40.4
Presence of any other animal species (yes; no) [a]	136; 35	64.0; 80.0	39.1; 58.9
By animal species (yes; no)			
- Dairy cattle (mean 56.7, range 1-160, med. 40)	18; 153	38.9; 70.6	
- Beef cattle (mean 19.1, range 1-60, med. 6.5)	9; 162	100.0; 65.4	
- Poultry (mean 1989, range 1-50000, med. 8)	26;145	46.2; 71.0	
- Sheep (mean 31.2, range 2-200, med. 15)	25; 146	68.0; 67.1	
- Goat (mean 5.1, range 1-30, med. 2)	10; 161	90.0; 65.8	
Hygiene score (≤ 3; 4-5; ≥6) [a, c]	30; 98; 43	53.3; 68.4; 74.4	42.9; 51.0; 55.8
Rodent control (self; professional)	100; 71	65.0; 70.4	41.0; 46.2
Fly control (yes; no) [a]	146; 25	70.6; 48.0	46.7; 22.8
Separate stables per age group (yes; no) [d]	64; 107	62.5; 70.1	41.0; 44.4
Separate clothing per age group (yes; no) [d]	23; 148	69.6; 66.9	47.0; 42.6
Separate tools per age group (yes; no) [d]	78; 93	71.8; 63.4	47.2; 39.7
Purchase of gilts (yes; no) [b]	98; 73	66.3; 68.5	38.7; 49.0
Housing sows (individual; small groups; large groups)	113; 33; 25	67.3; 66.7; 68.0	41.2; 48.2; 45.2
Washing sows (yes; no)	122; 49	68.9; 63.3	45.9; 36.3
Cross-fostering (<24 hr; > 24 hr; both)	96; 56; 19	65.6; 69.6; 68.4	41.1; 45.6; 46.3
Float in drinking water system (yes; no) [a]	78; 93	60.3; 73.1	37.5; 47.9
Adding acid to drinking water (yes; no; no finishing pigs)	16; 126; 29	75.0; 67.5; 62.1	57.5; 43.4; 34.1
Antimicrobial use (batch; individual; no) [a]	138; 27; 5	70.3; 51.9; 80.0	51.0; 40.5; 32.6

[a] Variables with $P < 0.20$ (-2LL) in univariable analysis on herd and pooled sample level

[b] Variables with $P < 0.20$ (-2LL) in univariable analysis on pooled sample level

[c] Hygiene score was classified as low (≤ 3 hygiene measures), medium (4-5 hygiene measures) and high (≥ 6 hygiene measures); following hygiene measures were scored: separate entrance and exit changing room; use of changing room by farmer; use of shower; use of water and soap; use of disinfection bath; separate clothing and tools per age group

[d] Pool prevalences are presented for all age groups together; analysis on pooled sample level was done for each group separately

status of finishing herds is highly associated with the MRSA status of their pig supplier (Broens et al., 2011c); (2) large differences in characteristics between breeding and finishing herds necessitated a separate analysis for both herd types decreasing the power of both risk analyses, and finally, (3) this enabled us to combine and compare our study with the EU-wide baseline survey on the prevalence of MRSA in holdings with breeding pigs (EFSA, 2009, 2010; Broens et al., 2011a). Using the formula to estimate prevalence (Noordhuizen et al., 2001) with an expected prevalence of 50% MRSA positive herds, a 95% confidence level and an absolute precision of 7.5%, 171 herds are needed to be sampled out of the total number of Dutch breeding herds (n = 3289) for an accurate prevalence estimation. Breeding herds were randomly selected from the total number of Dutch breeding herds. Protocols for sampling and information collection as well as microbiological analyses for the breeding herds in 2008 were identical to those in 2007. In total, 171 breeding herds were sampled: 19 in 2007 and 152 in 2008. The distribution of sampled herds over the 12 Dutch provinces was not different from the distribution of non-sampled herds (Chi-Square $P > 0.05$).

Herds were categorized according to regulations from the Dutch Product Boards for Livestock, Meat and Eggs. Breeding herds were divided into either category A (breeding holdings with strict control measures) or category B (production holdings with standard control measures), and all finishing herds were placed under category D. A total of 80 category A herds, 91 category B herds and 31 category D herds were sampled. The total number of sampled breeding herds (n = 171) represented 5.2% of all breeding herds in the Netherlands, 18% of all Dutch category A herds (n = 449) and 3% of all Dutch category B herds (n = 2840). The number of sows per herd was chosen as a measure for herd size; the average herd size in this study was 431 (median = 320; Q1-Q3: 220-500; range: 24-2100). As antimicrobial use has been suggested to be associated with the presence of MRSA, we classified antimicrobial use and analysed the results in 2 ways: (1) batch treatment / individual treatment / no treatment and (2) batch treatment with 'risk antimicrobials' / batch treatment with other antimicrobials or individual treatment with antimicrobials / no treatment. 'Batch treatment' was defined as preventive application of antimicrobials at specific time points (e.g. birth or weaning), most often applied to a group of pigs. 'Individual treatment' was defined as curative application of antimicrobials to individual pigs or a restricted group of pigs presenting clinical signs (e.g. coughing or scour). 'No treatment' was defined as no application of antimicrobials. Penicillins, cefalosporins (both β-lactams) and tetracyclins were defined as 'risk antimicrobials', as LA-MRSA is 100% resistant to these antimicrobial classes (Kadlec et al., 2009); all other antimicrobial classes were defined as 'other antimicrobials'.

To compare microbiological results from both laboratories (Labs I and II), 8 herds classified as MRSA positive were sampled again in 2009. Two nasal swabs (A and B) were simultaneously taken from the same nostril in 60 pigs, and 10 environmental wipes were taken in 5 pairs (A and B) from adjacent surfaces. All samples labelled 'Sample A' were sent to Lab I, while those labelled 'Sample B' were sent to Lab II for analysis (see below for detailed laboratory procedure).

Microbiological analyses

All samples were transported to the lab at day of sampling. Samples were stored at 4°C until processing, which occurred within 10 days after sampling according to guidelines formulated by the European Food Safety Authority (EFSA, 2007). Average number of days (± SD) between sampling and processing was 2.8 (± 2.4). MRSA was isolated from both single environmental wipes and pooled nasal swabs following the protocol described by Broens et al. (2010). Briefly, double enrichment in selective broths (Mueller Hinton Broth with 6.5% NaCl and Phenol Red Mannitol Broth with 4 mg/L ceftizoxime and 75mg/L aztreonam (BioMérieux, NL020, France) was followed by plating on a chromogenic MRSA screen agar (Oxoid, PO5196A, United Kingdom). One suspected colony per sample was then confirmed by multiplex PCR (De Neeling et al., 1998; Martineau et al., 1998) and typed by *spa* typing (Harmsen et al., 2003). Multilocus sequence typing (Enright et al., 2000) was performed on 1 isolate for each *spa* type.

Statistical analysis

All statistical analyses were performed using the SAS version 9.1 software (SAS, 2004). A herd was classified MRSA positive if ≥ 1 sample (pooled or environmental) tested positive. Exact confidence intervals (CIs) were calculated based on the binomial distribution (PROC FREQ). Cohen's kappa, a measure of agreement (Cohen, 1960), was calculated to compare results from both laboratories (PROC FREQ).

Risk factor analysis was done for breeding herds only. Herd characteristics were used to define variables for risk factor analysis (Table 1), which was performed at both the level of herds (171 breeding herds) and of pooled samples (1699 pooled samples from 170 breeding herds); the origin of 10 pooled samples was missing from 1 herd, and 1 pooled sample was missing from another herd. Analysis of pooled samples enabled us to analyse explanatory variables known for a specific age group, as several variables, e.g. antimicrobial use, hygiene and drinking water system, were recorded per age group sampled. First, potential factors associated with MRSA on herds and pooled samples were identified using univariable logistic regression analysis (PROC LOGISTIC). Variables with *P*-values < 0.20 based on -2 log likelihood were further used in multivariable analysis. As our study spanned only 2 years, the variables 'period' and 'season' were highly correlated with MRSA prevalence. In addition, the number of herds sampled per time period varied widely, ranging from only 5 herds in the second half of 2007 to 91 herds in the first half of 2008 (Table 1). Therefore, time (in months of study) was included in our model. The linearity of the logits for herd size (number of sows) and time (month) were assessed and included as continuous explanatory variables. A backward elimination procedure was performed for multivariable analysis according to the method described by Hosmer and Lemeshow (1989), in which the least significant variable was eliminated stepwise from the model until all remaining variables had a $P <$ 0.05 or were confounders. Confounding was checked by monitoring the change in regression parameters (β) and was considered to be present if $\Delta\beta > 25\%$ or $\Delta\beta > 0.1$, if $-0.4 < \beta < 0.4$. For multivariable analysis on pooled samples, a random

herd effect was included in the model to adjust for samples from the same herd that might not be independent (PROC GLIMMIX); an exchangeable covariance structure was used to account for within-herd variation. In the final multivariable models, interaction terms between variables were tested for significance based on the likelihood ratio test ($P < 0.05$). The strength of association between a variable and MRSA prevalence is presented in terms of odds ratios (OR; Noordhuizen et al., 2001). General linear regression (PROC GLM) was performed to assess whether potential risk factors were associated with herd size.

RESULTS

Descriptive statistics

Of 31 finishing herds, 22 were classified MRSA positive (71.0%; 95% CI: 52.0-85.8). On average, 53.6% of the pooled samples (median = 55; Q1-Q3: 20-90) and 57.3% of the environmental samples (median = 60; Q1-Q3: 20-100) tested positive for MRSA in finishing herds.

Of 171 breeding herds, 115 were classified MRSA positive (67.3%; 95% CI: 59.7-74.2). On average, 63.8% of the pooled samples (median = 70; Q1-Q3: 50-80) and 33.9% of the environmental samples (median = 20; Q1-Q3: 0-60) tested positive for MRSA in breeding herds. Focusing on the age of pigs present in the breeding herds, 43.4% of the pooled samples were from sows, 19.0% from suckling piglets, 22.5% from weanling piglets, 8.7% from replacement pigs and 6.5% from finishing pigs. The overall prevalence of MRSA in pooled samples from breeding herds was 43.2% (95%CI: 40.8-45.6), with the highest MRSA prevalence observed among suckling (53.4%) and weanling (52.9%) piglets and the lowest in replacement pigs (23.8%) (Table 3a).

The majority of MRSA positive finishing and breeding herds (70.1%) had at least 1 pooled and 1 environmental sample that tested positive for MRSA, while other herds tested positive in either pooled (24.8%) or environmental samples (5.1%), but not both. Finishing herds were more often positive based on only environmental samples (18.2% of positive finishing herds) compared to breeding herds (2.6%). Conversely, breeding herds were more often positive based on only pooled samples (27.0% of positive breeding herds) compared to finishing herds (13.6%), though this was not significantly different for finishing and breeding herds (Chi-Square $P > 0.05$).

To compare results between laboratories, 8 herds were resampled in 2009 and found to be MRSA positive in both laboratories. Lab I found that 72.5% (58/80) of the pooled samples and 50.0% (20/40) of the environmental samples were positive for MRSA, while Lab II found that 57.5% (46/80) of the pooled samples and 52.5% (21/40) of the environmental samples were MRSA positive. The Cohen's kappa between laboratories was 0.57 when comparing all samples (95% CI: 0.42-0.72), indicating moderate agreement between both labs. Agreement among pooled samples ($\kappa = 0.63$; 95% CI: 0.46-0.79) was higher than that among environmental samples ($\kappa = 0.45$; 95% CI: 0.17-0.73).

Molecular typing

Spa typing was performed on 1110 MRSA isolates: 929 isolates originated from 115 MRSA positive breeding herds, and 181 isolates originated from 22 MRSA positive finishing herds. A total of 19 spa types were detected: 17 *spa* types (1091 isolates) were confirmed to belong to CC398 (Table 2), while the other 2 *spa* types, t002 and t127 belonged to ST5 and ST1, respectively. Nine *spa* types were isolated from both finishing and breeding herds, whereas the other 10 *spa* types were isolated from breeding herds only. *Spa* types t011 and t108 were the most frequently found, accounting for 50.1% and 35.9% of the total *spa* types observed in our study. Only 1 *spa* type was found in 99 herds (72.3% of MRSA positive herds), 2 *spa* types were found in 27 herds (19.7%) and 3 *spa* types were found in 11 herds (8.0%).

Table 2. Identified *spa* types of 1110 MRSA isolates, Multi Locus Sequence Type, frequency (*n* and %) and number of (breeding / finishing) pig herds in which the *spa* types were found in The Netherlands.

Spa type	MLST	Frequency		Number of herds	
		n	%	Breeding	Finishing
t011	ST-398	556	50.1	71	12
t108	ST-398	398	35.9	52	10
t1457	ST-398	37	3.3	9	0
t899	ST-398	20	1.8	3	1
t567	ST398	18	1.6	2	2
t127	ST-1	14	1.3	2	0
t1184	ST-398	13	1.2	4	1
t571	ST-398	11	1.0	1	1
t2330	ST-398	10	0.9	1	1
t1456	ST-398	8	0.7	2	0
t002	ST-5	5	0.5	1	0
t2346	ST-398	5	0.5	1	1
t034	ST-398	4	0.4	1	1
t588	ST-398	4	0.4	1	0
t3479	ST-398	3	0.3	1	0
t943	ST-398	1	0.1	1	0
t1451	ST-398	1	0.1	1	0
t2011	ST-398	1	0.1	1	0
t4119	ST-398	1	0.1	1	0

Risk factor analysis

After univariable analysis on herds, the following variables had *P*-values < 0.20: time, herd size, herd category, presence of finishing pigs, presence of other animals, pig density region, hygiene score, fly control, float in drinking water system and antimicrobial use (Table 1). After performing backward elimination, only 2 variables remained in the final model (time and herd size), and no interaction was observed between these variables ($P > 0.05$). Herd prevalence of MRSA increased in time (OR = 1.09 per month; $P = 0.015$), from ~30% in the first quarter of 2007 to ~75% in the last quarter of 2008 (Fig. 1). The percentage of positive pools within MRSA positive herds varied over time (Fig. 1). In addition, MRSA prevalence increased with herd size (OR = 1.32 per 100 sows; $P = 0.002$), as ~40% of herds with < 250 sows were MRSA positive, whereas > 80% of herds with > 500 sows were MRSA positive (Fig. 2). The percentage of MRSA positive pooled samples in herds also appeared to increase with herd size from ~50% in herds with < 250 sows to ~75% in herds with > 500 sows (Fig. 2). To visualize the effect of time and herd size, we performed univariable regression analysis (shown in Figs. 1 and 2). Elimination of either variable from the multivariable analysis did not noticeably affect the results of univariable regression analysis.

In addition to the 10 variables used to analyse herds, purchase of gilts and age group were also included in our multivariable analysis for pooled samples. After performing backward elimination, 3 variables with a $P < 0.05$ remained, and no significant interaction ($P > 0.05$) was present between these variables (Tables 3a and b). Adding a random herd effect did change the estimates and their 95% confidence intervals, but the direction of the effects remained unchanged. In line with results for herd level analysis, the prevalence of MRSA in pooled samples increased with time (OR = 1.10 per month; $P = 0.025$) and herd size (OR = 1.31 per 100 sows; $P < 0.001$). Furthermore, age group was associated with MRSA prevalence in pooled samples (overall $P < 0.0001$), as the MRSA prevalence in pooled samples from suckling and weanling piglets (53.4% and 52.9%, respectively) was higher than that in sows (38.3%; $P < 0.0001$); the MRSA prevalence in replacement pigs was lower (23.8%; $P < 0.0001$) than that in sows (Table 3a). The potential and frequently suggested association between antimicrobial use and MRSA prevalence led us to add the variable 'antimicrobial use' (despite having a $P > 0.05$) in our final model. Elimination of 'antimicrobial use' from both final models had hardly an effect on the regression parameters of the other variables (< 10% change). Though overall not significant ($P = 0.1396$), pooled samples from batch-treated pigs (51.0%; $P = 0.05$) as well as from individually treated pigs (40.5%; $P = 0.14$) were more often found to be MRSA positive than pooled samples from untreated pigs (32.6%; Table 3a). Similarly, for risk antimicrobials, batch-treated pigs (55.5%; $P = 0.25$) and pigs receiving other antimicrobial treatments (41.3%; $P = 0.04$) were found more often to be MRSA positive than untreated pigs (32.6%). Again, overall not statistically significant ($P = 0.1016$; Table 3b). Antimicrobial use varies between different age groups. Batch treatments were more often applied to suckling (65.5%) and weanling piglets (53.7%) than to sows (29.3%), finishing pigs (21.6%) or replacement pigs (8.8%; $P < 0.0001$; Table 4). Batch treatments specifically with risk antimicro

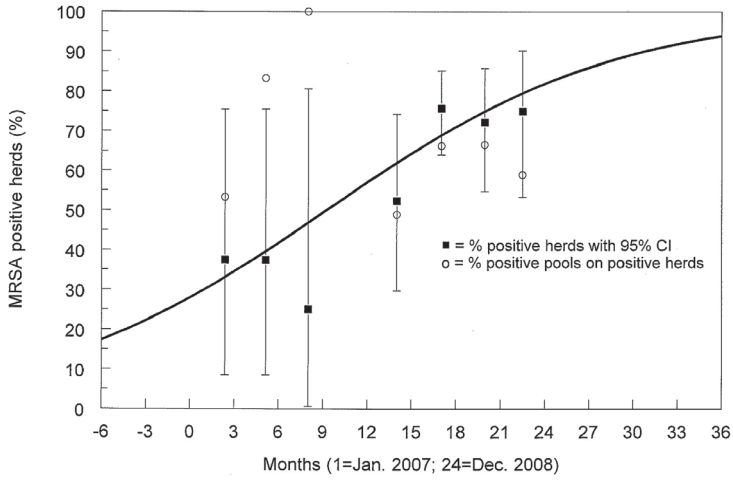

Figure 1. Percentage MRSA positive herds and percentage positive pooled samples on MRSA positive Dutch pig herds in time (in months; start study in month 1). The logistic regression line represents the estimated probability of a herd being MRSA positive given the month of study: $1/(1 + \exp^{(0.9456 - 0.1024 \times \text{month})})$

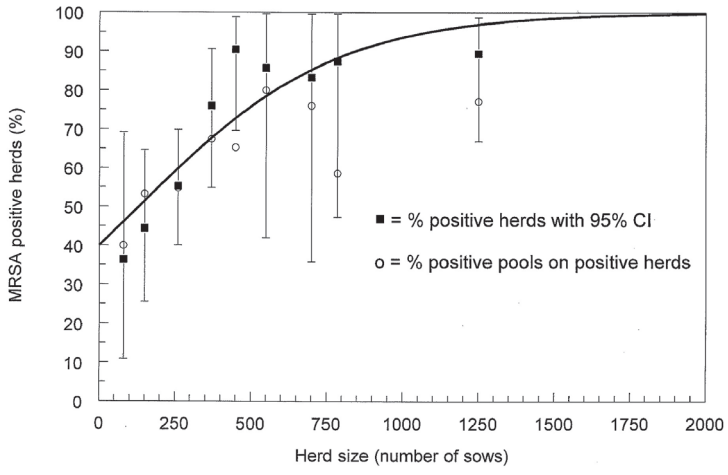

Figure 2. Percentage MRSA positive herds and percentage positive pooled samples on MRSA positive Dutch pig herds related to herd size (in number of sows). The logistic regression line represents the estimated probability of a herd being MRSA positive given the herd size: $1/(1 + \exp^{(0.4053 - 0.0031 \times \text{number of sows})})$

Table 3. Variables and their Odds Ratio with 95% confidence interval in the final multivariable model for a pooled sample to be MRSA positive in Dutch pig herds with correction for random herd effect (*n*=1699).

Variable	Category	Frequency		MRSA (%)	OR	95% CI	*P*-wald	Overall *P*
		n	%					
(a)								
n sows (per 100)	Continuous				1.31	1.17-1.46	< 0.0001	
Time (per month)	Continuous				1.10	1.01-1.19	0.0245	
Age group	Replacement pigs	147	8.7	23.8	0.23	0.13-0.41	< 0.0001	< 0.0001
	Finishing pigs	111	6.5	38.7	1.82	0.93-3.56	0.0809	
	Weanling piglets	382	22.5	52.9	4.77	3.17-7.17	< 0.0001	
	Suckling piglets	322	19.0	53.4	3.86	2.50-5.97	< 0.0001	
	Sows	737	43.4	38.3	Ref.	Ref.	Ref.	
Antimicrobial use	Batch	669	39.4	51.0	1.80	1.01-3.21	0.0473	0.1396
	Individual	726	42.7	40.5	1.56	0.87-2.79	0.1392	
	No	304	17.9	32.6	Ref.	Ref.	Ref.	
(b)								
n sows (per 100)	Continuous				1.31	1.17-1.46	< 0.0001	
Time (per month)	Continuous				1.10	1.01-1.19	0.0241	
Age group	Replacement pigs	147	8.7	23.8	0.23	0.13-0.41	< 0.0001	< 0.0001
	Finishing pigs	111	6.5	38.7	1.79	0.91-3.51	0.0921	
	Weanling piglets	382	22.5	52.9	4.79	3.21-7.16	< 0.0001	
	Suckling piglets	322	19.0	53.4	4.06	2.59-6.37	< 0.0001	
	Sows	737	43.4	38.3	Ref.	Ref.	Ref.	
Antimicrobial use	Risk-batch	418	24.6	55.5	1.43	0.77-2.66	0.2542	0.1016
	Other	977	57.5	41.3	1.83	1.04-3.22	0.037	
	No	304	17.9	32.6	Ref.	Ref.	Ref.	

Batch = batch treatment with antimicrobials; ind = individual animal treatment with antimicrobials; risk = risk-antimicrobials (= tetracycline and β-lactams)

bials were most often applied to suckling and weanling piglets (56.2% and 29.3%, respectively; $P < 0.0001$).

Table 5 lists variables with $P < 0.20$ from univariable analysis that were associated with herd size ($P < 0.05$). We found that category A herds (570 sows) were larger than category B herds (310 sows), herds that had purchased gilts (511 sows) were larger than those that do not purchase gilts (373 sows) and that herds with fly control (457 sows) were larger than those without fly control (288 sows). Moreover, herd size also increased with hygiene score, as low hygiene herds (≤ 3 measures) were smaller (323 sows), than medium hygiene herds (419 sows) and high hygiene herds (≥ 6 measures; 535 sows). While herds that applied batch treatments with antimicrobials (459 sows) were larger than those that used individual treatments (320 sows), herds receiving batch treatments with risk antimicrobials were the largest (467 sows). However, due to the low number of herds that did not use antimicrobials at all ($n = 5$), the average herd size in this category (301 sows) was not significantly different from the other categories with respect to antimicrobial use.

Table 4. Percentage of pooled samples taken in Dutch pig herds where antimicrobials were used per age group.

Age group	Antimicrobial use	
	Batch (%)	Risk-batch (%)
Sows	29.3	15.3
Suckling piglets	65.5	56.2
Weaning piglets	53.7	29.3
Replacement pigs	8.8	3.4
Finishing pigs	21.6	6.3
Total	39.4	24.6

Batch = batch treatment with antimicrobials, risk-batch = batch treatment with risk-antimicrobials (= tetracycline and β-lactams)

Table 5. Least square (LS) means herd size (number of sows) with standard error of mean (SEM) of Dutch pig herds included in the multivariable analysis per category of variables that were related to herd size.

Variable	Category	LS means herd size	SEM	*n*
Herd category	A	570 [a]	37	90
	B	310 [b]	35	80
Purchase of gilts	yes	511 [a]	41	73
	no	373 [b]	35	97
Fly control	yes	457 [a]	70	145
	no	288 [b]	29	25
Presence of other animals	yes	401 [a]	30	135
	no	554 [b]	59	35
Presence of finishing pigs	yes	355 [a]	45	62
	no	476 [b]	34	108
Float in drinking water system	yes	345 [a]	39	77
	no	503 [b]	36	93
Pig density in region	> 17 pigs/ha	465 [a]	39	83
	≤ 17 pigs/ha	401 [b]	38	87
Hygiene score	low	323 [a]	65	29
	medium	419 [b]	35	98
	high	535 [c]	53	43
Antimicrobial use (a)	batch	459 [a]	157	138
	individual	320 [b]	68	27
	no	301 [ab]	30	5
Antimicrobial use (b)	risk-batch	467 [a]	157	118
	other	358 [b]	51	47
	no	301 [ab]	32	5

[abc] Different superscript within a variable indicates a significant difference ($P < 0.05$; general linear regression analysis)

DISCUSSION

In this study, we found that the prevalence of MRSA positive herds was 67.3% in breeding herds and 71.0% in finishing herds, which is higher than previously reported for pig herds in the Netherlands (Van Duijkeren et al., 2008; EFSA, 2009). In 2007, Van Duijkeren et al. (2008) reported an MRSA prevalence of 23% from sampling 10 pigs in 31 herds. However, with a sample size of 10 pigs per herd, MRSA can only be detected when the within-herd prevalence exceeds 25% (Noordhuizen et al., 2001). In our study, we sampled 60 pigs per herd, meaning that MRSA can be detected when the within-herd prevalence exceeds 5%, assuming that pooling of nasal swabs has a negligible effect on detection sensitivity. In 2008, an EU-wide baseline survey collected 5 environmental samples from each breeding herd and pooled these samples for MRSA detection. The apparent prevalence of MRSA positive pig herds in the Netherlands was 17% (EFSA, 2009). However, pooling environmental samples appeared to largely affect their analysis, resulting in a substantial underestimation of the true prevalence (Broens et al., 2011a); this might explain the significantly higher prevalence observed in our study compared to the EU-wide baseline survey. On the other hand, we might have overestimated the prevalence of MRSA positive herds by ~5% because we sampled nearly equal numbers of category A and B herds when, in fact, the number of category B herds in the Netherlands is ~6 times higher than that of category A herds.

The number of MRSA positive herds increased during the study period. Extrapolation of our data implies that nearly all Dutch pig herds might become MRSA positive within a few years. This increase cannot be explained simply by different laboratory environments, as both laboratories gave similar results for herds. Furthermore, Lab I reported higher MRSA prevalences for pooled samples than Lab II, making it even more unlikely that laboratory environments could have caused the increase in MRSA prevalence. The most likely explanation for the observed increase in MRSA positive herds is that MRSA is easily transmitted between herds (e.g. when purchasing animals) (Broens et al., 2011c).

Herd size was shown to be highly associated with MRSA, which was also shown in an EU-wide survey in holdings with breeding pigs (EFSA, 2010). A similar association was found in surveys on fattening pig herds and veal farms (Tenhagen et al., 2009; Battisti et al., 2010; Graveland et al., 2010). For several other infectious pig diseases, such as *Salmonella enterica* in pigs, the effect of herd size on disease prevalence has been reported (Carstensen and Christensen, 1998). Possible explanations for a positive association between herd size and MRSA prevalence might be due to a higher risk of introduction into a larger herd, a higher number of susceptible animals either by birth of purchase of pigs, or a higher probability of persistence in a larger herd, which has been described for other swine diseases (Gardner et al., 2002; Evans et al., 2010). Differences in herd management might also contribute to an effect of herd size, which was shown in our multivariable analysis. Although the effect of each individual management variable (purchase of gilts, hygiene score and antimicrobial use) was too small

to yield a significant effect on MRSA prevalence, a significant association was observed between each variable and herd size. Herd size appears to be an accumulation of several risk factors and significantly affects the prevalence of LA-MRSA.

Age group was also shown to be associated with MRSA. Pooled samples from suckling and weanling piglets were more often MRSA positive than those from sows, finishing pigs or replacement pigs, possibly due to the higher overall susceptibility to infection or greater impact of antimicrobials on the unbalanced microbiota of young piglets (Zoetendal et al., 2004b; Bailey et al., 2005). In fact, our data show that suckling and weanling piglets are more often treated with antimicrobials than sows, finishing pigs and replacement pigs.

Studies in humans show an association between antimicrobial use and the occurrence of MRSA (Beam and Buckley, 2006; Muller et al., 2006; Dancer, 2008), and batch-treated calves were more often MRSA positive than untreated calves (Graveland et al., 2010). The same association was suggested for pig husbandry (De Neeling et al., 2007; Van Duijkeren et al., 2008), but has not yet been confirmed. Univariable and multivariable analyses (without correcting for a random herd effect) showed that antimicrobial use was indeed associated with MRSA prevalence, which was the highest in pooled samples from pigs exposed to antimicrobials (51.0%) and lowest in pooled samples from unexposed pigs (32.6%). However, after correcting for herd effect in the final multivariable model, no significant association was found between antimicrobial use and MRSA prevalence of pooled samples.

The 2 predominant *spa* types in this study, t011 and t108, have been frequently observed in other Dutch studies (De Neeling et al., 2007; Van Duijkeren et al., 2008; Huijsdens et al., 2009) and accounted for approximately 63% of MRSA isolates reported in an EU-wide baseline survey (EFSA, 2009). However, *spa* type t108 is relatively more abundant in the Netherlands than in other member states. *Spa* type t1457 is localized primarily in the north eastern part of the Netherlands (data not shown), making it worthwhile to look for a common source. *Spa* types t002 and t127 do not belong to CC398 but have been previously reported to be found in humans, pigs and horses (Cuny et al., 2008; Khanna et al., 2008; SWAB, 2009).

CONCLUSIONS

In this study, we show that the majority of pig herds in the Netherlands are positive for LA-MRSA, and prevalence is steadily increasing over time. We found that larger herds are more likely to be MRSA positive than smaller herds, possibly due to a higher risk of introduction and an increased probability of persistence. However, multiple factors affecting MRSA prevalence are also positively (though not significantly) associated with herd size (e.g. antimicrobial use, purchase of gilts and hygiene level), indicating that herd size is a compilation of risk factors that increases the likelihood for larger herds to be MRSA positive.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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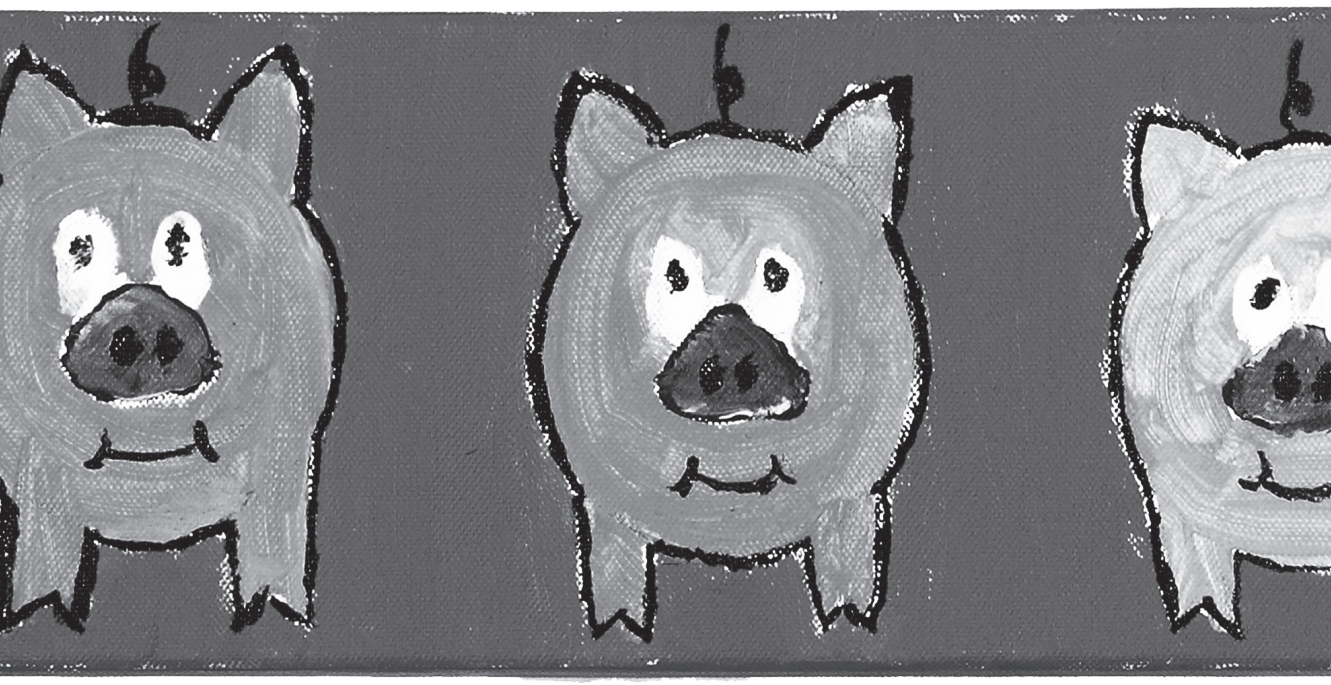
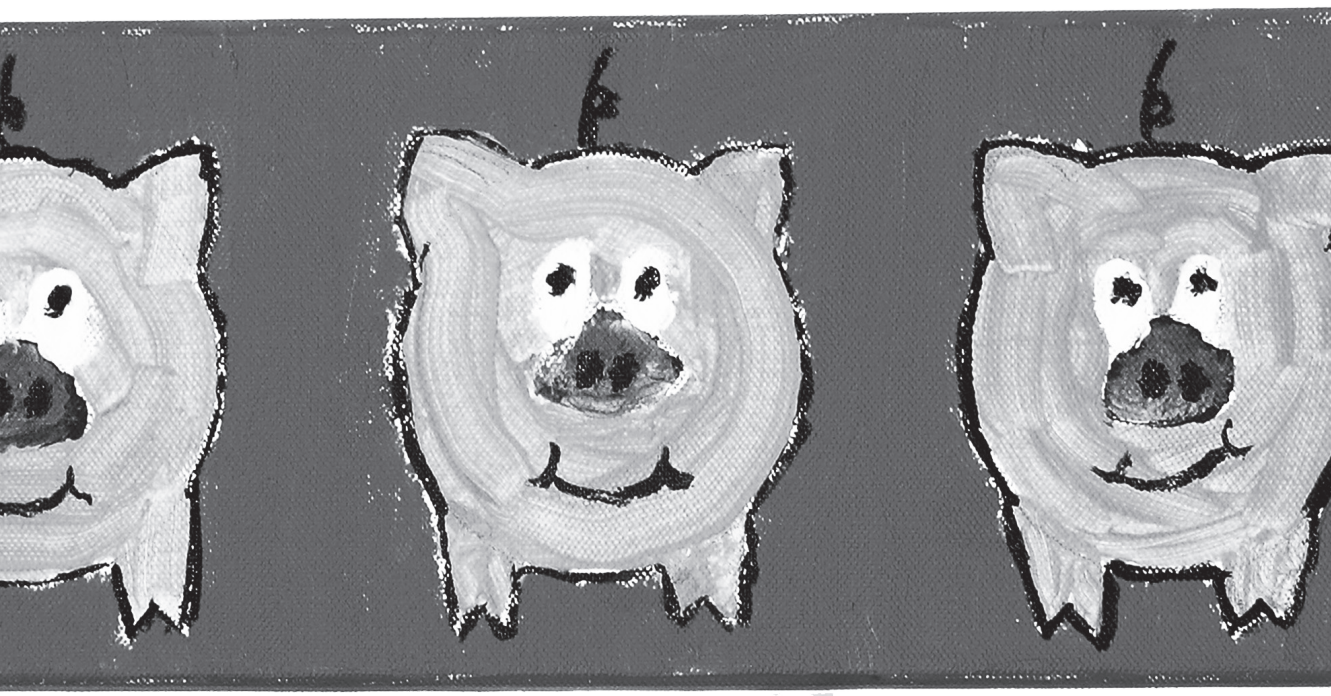
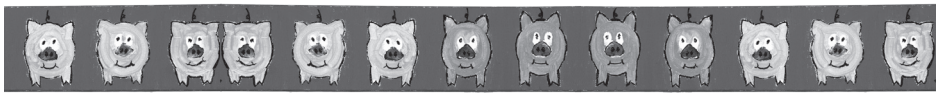


PART III – LA-MRSA IN THE SLAUGHTERHOUSE



CHAPTER 3.1

TRANSMISSION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AMONG PIGS DURING TRANSPORTATION FROM FARM TO ABATTOIR



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KEYWORDS

- Methicillin resistant *Staphylococcus aureus* (MRSA)
- Pigs
- Transmission
- Transportation
- Abattoir

ABSTRACT

The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in pigs at abattoirs is higher than in pigs sampled on farms. This study investigated whether MRSA negative pigs can become MRSA positive during transportation from the farm to the abattoir after exposure to other pigs and environmental sources of MRSA. Nasal swabs were collected from four batches of pigs during loading at the farm, on arrival at the abattoir and after stunning. Environmental wipes were taken from lorries after transporting pigs and from lairages after holding pigs. All pigs (n = 117) tested MRSA negative before transportation. On arrival at the abattoir, 12/117 (10.3%) pigs in two batches tested MRSA positive. In lorries that tested positive after transportation, the prevalence of MRSA positive pigs was 21.1%, whereas no MRSA was detected in pigs that had been transported in lorries that tested negative after transportation. At stunning, all batches and 70/117 (59.8%) pigs tested MRSA positive. Pigs can become MRSA positive in the short period of time during transportation from the farm to stunning at the abattoir.

INTRODUCTION

In 2005 in The Netherlands, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA CC398) was found in pigs and in humans who had been in contact with pigs (Voss et al., 2005). Since then, this clone has been identified in several countries in both pigs and other livestock (Khanna et al., 2008; EFSA, 2009; Smith et al., 2009; Van Den Broek et al., 2009; Wagenaar and Van De Giessen, 2009; Mulders et al., 2010). In a study performed in Dutch abattoirs, 209/540 (39%) pigs and 44/54 (81%) slaughter batches were MRSA positive in 2005–2006 (De Neeling et al., 2007). In another Dutch study on pig farms using similar diagnostic methods, 7/31 (23%) farms and 35/310 (11%) pigs were MRSA positive in 2006 (Van Duijkeren et al., 2008).

The higher prevalence of MRSA in pigs in abattoirs compared to the prevalence on farms might be due to MRSA transmission at abattoirs (De Neeling et al., 2007) or during transportation to the abattoir. For *Salmonella enterica* serovar Typhimurium, a short exposure to contaminated environments (such as lorries and lairages in abattoirs) is sufficient to result in positive pigs (Hurd et al., 2001; Boughton et al., 2007). The objective of the present study was to determine whether pigs become positive for MRSA CC398 during transportation from the farm to the abattoir and while being held in lairages at the abattoir.

MATERIALS AND METHODS

Study design

From July 2008 to April 2009, four MRSA negative farrow-to-finish farms (farms A–D) were selected out of a national prevalence and risk factor survey on MRSA (Wagenaar and Van De Giessen, 2009). In this survey, 60 nasal swabs from pigs (in 10 pools of six swabs each) and five environmental wipes were tested for the presence of MRSA. A farm was classified as MRSA negative if all samples tested negative. Information on the selected farms is presented in Table 1.

After transportation, lorries were cleaned using high pressure water, followed by disinfection, according to Dutch guidelines for animal transportation (Anonymous, 2005). The time of transportation ranged from 2 to 5 h. On the way to the abattoir, pigs from other farms were picked up by the same lorry for batches A, B and C. Pigs from other farms were located in separate lorry sections, but contact between pigs from different farms was possible, either directly by nose-to-nose contact between pigs in different lorry sections or indirectly through contact with excreta. Slaughter pigs from the four farms were transported to three different commercial abattoirs (abattoirs I–III).

The floors of all lairages were constructed of concrete with rough surfaces, as were the separation walls between lairage sections in abattoir II. In the other two abattoirs, open metal fences separated the lairage sections. Production units in all abattoirs, including the lairages, were cleaned at the end of every working day using high pressure water, followed by alkaline or acid disinfectants. Lairages were disinfected twice weekly. The killing method in all abattoirs was electrical

stunning, followed by bleeding.

Pigs were held in lairages for 1.75 – 11.5 h. During this time period, there were frequent movements of pigs in and out of the lairages, due to delivery of pigs from other farms, while others were removed for slaughter. Pigs from other farms were located in separate lairage sections, but contact between pigs from different farms was possible, directly or indirectly, particularly in the abattoirs with open fencing.

Table 1. Information on farms, abattoirs, transportation and positive samples at loading, on arrival and at stunning.

	Batch A	Batch B	Batch C	Batch D
Farm size (number of sows)	160	160	320	380
Abattoir	I	II	III	III
Pick up of pigs during transportation	Yes	Yes	Yes	No
Transport time in lorry (h)	5	5	4	2
Holding time in lairage (h)	9	1.75	11.5	2
Total time (h)	14	6.75	15.5	4
Number of pigs present in lairage	~1000	~500	~800	~800
Type of fencing in lairage	Open	Closed	Open	Open
Number of pigs in batch	60	65	27	63
Sex of pigs (M, male; F, female)	M and F	M	M	F
Number of pigs tested	30	30	27	30
Number of positive pigs at loading	0	0	0	0
Number (%) positive pigs on arrival	0 (0.0)	0 (0.0)	7 (25.9)	5 (16.7)
Number (%) positive pigs at stunning	2 (6.7)	13 (43.3)	27 (100.0)	28 (93.3)
Positive/Total wipes lorry[a]	0/3	0/5	1/5	1/5
Positive/Total wipes lairage[a]	0/3	4/5	1/5	1/5

[a] Environmental wipes were taken after transportation in lorries and holding in lairages.

Sampling from pigs, lorries and lairages

Nasal swabs (MW102, Medical Wire and Equipment) were taken from 27 to 30 slaughter pigs at the following time points: (1) at the farm just before loading; (2) on arrival at the abattoir; and (3) just after stunning; pigs were not restrained for sampling and the same pigs were sampled at each of the three time points. Environmental wipes (s1 Kit Ringer Solution, Sodibox) were taken from lorries after transportation and from lairages after holding pigs.

Isolation and typing of MRSA

All samples were sent to the Animal Health Service, Deventer, The Netherlands, for analysis within 10 days of sampling. Samples were first enriched using Mueller Hinton Broth with 6.5% NaCl (MHB+). Nasal swabs were put into 10 mL MHB+ and environmental wipes into 100 mL MHB+. After 18 h incubation at 37 °C, 1 mL MHB+ was transferred into 9 mL phenol red mannitol broth containing 75 mg/L aztreonam and 4 mg/L ceftizoxime (PMB+; NL020, BioMérieux), which was incubated for a further 18 h at 37 °C. A loopful of PMB+ was spread onto sheep blood agar (PB5008A, Oxoid) and a chromogenic MRSA screen agar (PO5196A, Oxoid), then incubated for a further 18 h at 37 °C. One suspected colony per sample was confirmed as MRSA by multiplex PCR (De Neeling et al., 1998; Martineau et al., 1998) and *spa* typed (Harmsen et al., 2003).

Statistical analysis

Data were analysed using SAS version 9.1 (SAS, 2004). Confidence intervals (CIs) for prevalences were calculated based on the binomial probability function (PROC FREQ). Because of the limited number of batches and therefore potential entanglement between batch and explanatory variables (MRSA status of environment, fencing type, lorry, abattoir and holding time), and since environmental samples were taken after pigs had been removed from lorries and lairages, a Generalised Estimating Equations model was performed, with batch as a random effect to solely estimate the clustering effect using an exchangeable covariance structure (PROC GENMOD).

RESULTS

Detection of MRSA in samples from pigs, lorries and lairages

All pigs (n = 117) tested MRSA negative before transportation (Table 1). On arrival at the abattoir, 12/117 (10.3%) pigs tested positive for MRSA; 7/27 (25.9%) pigs were positive from batch C and 5/30 (16.7%) pigs were positive from batch D, whereas all pigs from batches A and B were negative. In the lorries transporting batches C and D, MRSA was isolated from 1/5 environmental wipes after transportation of pigs, whereas no MRSA was detected in lorries after transporting batches A and B.

At stunning, 70/117 (59.8%) pigs were MRSA positive; positive pigs were found in all batches and the MRSA prevalence within each batch ranged from 2/30 (6.7%) for batch A to 27/27 (100.0%) for batch C (Table 1). MRSA positive environmental wipes were found in three lairages after holding pigs, ranging from 1/5 positive wipes for batches C and D to 4/5 positive wipes for batch B, whereas the lairage holding batch A tested negative (0/3).

The MRSA prevalence in pigs transported in lorries that tested positive after transportation was 21.1%, whereas MRSA was not detected in pigs transported in lorries that tested negative after transportation (Table 2). Mixing batches of pigs with pigs from other farms during transportation did not appear to increase MRSA

Table 2. MRSA status of pigs on arrival at the abattoir and at stunning.

Variable	Category	Frequency (*n*)	MRSA positive pigs (%)	95% CI
On arrival (all batches)				
MRSA status of lorry[a]	Positive	57	21.1	11.4-33.9
	Negative	60	0.0	0.0-6.0
Pigs picked up from other farms	Yes	87	8.0	3.3-15.9
	No	30	16.7	5.6-34.7
At stunning (all batches)				
MRSA status of lairage[a]	Positive	87	78.2	68.0-86.3
	Negative	30	6.7	0.8-22.1
Fencing	Open	30	43.3	25.5-62.6
	Closed	87	65.5	54.6-75.4
MRSA status of pigs on arrival	Positive	12	100.0	73.5-100.0
	Negative	105	55.2	45.2-65.0
MRSA status of lorry[a]	Positive	57	96.5	87.9-99.6
	Negative	60	25.0	14.7-37.9
At stunning (MRSA negative batches on arrival)				
MRSA status of lairage[a]	Positive	30	43.3	25.5-62.6
	Negative	30	6.7	0.8-22.1

[a] Based on environmental wipes that were taken after transport and resting time, respectively.

Table 3. *Spa* types from 90 MRSA isolates from pigs and the environment.

Spa type	Batch A	Batch B		Batch C				Batch D				
	S	S	La	A	Lo	S	La	A	Lo	S	La	Total
t011		6	3	3		18	1			22	1	54 (60%)
t108		6	1	4	1	8				3		23 (26%)
t1457	2							5	1	3		11 (12%)
t2123						1						1 (1%)
t2330		1										1 (1%)

A = pigs on arrival; S = pigs at stunning; Lo = lorry environment; La = lairage environment.

transmission; the MRSA prevalence was 8.0% (95% CI 3.3–15.9) in pigs transported together with pigs from other farms, compared to 16.7% (95% CI 5.6–34.7) in pigs transported without pigs from other farms. The MRSA prevalence did not appear to increase with increasing transport time, since both negative batches at arrival had the longest transport time (Table 1).

The MRSA prevalence in pigs from lairages that tested positive after holding pigs was 78.2% (95% CI: 68.0–86.3) compared to 6.7% (95% CI: 0.8–22.1) in pigs from lairages that tested negative after holding pigs (Table 2). All pigs that were MRSA positive on arrival were also MRSA positive at stunning, whereas 55.2% of pigs that were negative on arrival were MRSA positive at stunning. The MRSA prevalence in pigs at stunning that were transported in lorries that tested positive after transportation was 96.5% (95% CI: 87.9–99.6) compared to 25.0% (95% CI: 14.7–37.9) in pigs that were transported in lorries that tested negative after transportation.

The MRSA prevalence in pigs held in lairages with open fencing was 43.3% (95% CI: 25.5–62.6) compared to 65.5% (95% CI: 54.6–75.4) in pigs that were held in lairages with closed fencing. The MRSA prevalence in pigs did not appear to be related to holding time in lairages (Table 1). In MRSA negative batches after transport (batches A and B), the MRSA prevalence in pigs that were held in lairages that tested positive was 43.3% (95% CI: 25.5–62.6) compared to 6.7% (95% CI: 0.8–22.1) in pigs that were held in lairages that tested negative after holding pigs.

Spa typing of MRSA isolates

One MRSA isolate from every positive nasal and environmental sample was *spa* typed ($n = 90$). Five *spa* types belonging to CC398 were identified, all previously detected in pigs (Huijsdens et al., 2009; Table 3). The most frequent *spa* types were t011 (60.0%) and t108 (25.6%).

DISCUSSION

This study demonstrated that pigs that test negative can become MRSA positive in the short time period during transport to the abattoir and while being held in the lairage. Our results support the findings of De Neeling et al. (2007), who found a high prevalence of MRSA CC398 in pigs sampled at stunning in the abattoir and suggested that this might be due to exposure to MRSA in lorries and lairages. Pigs can become positive for *Salmonella enterica* serovar Typhimurium within 2 h of exposure to a contaminated environment (Hurd et al., 2001; Boughton et al., 2007).

The most likely sources of MRSA for pigs in lorries and lairages are the environment and pigs from other farms. In batch B, which was negative on arrival at the abattoir, 43.3% of pigs tested positive at stunning after being held in a lairage that tested positive afterwards. Moreover, this lairage had closed fencing, minimising contact with pigs from other batches, indicating that the lairage was a potential source of MRSA for these pigs. The source of MRSA for pigs in other batches may have been pigs within the batch (batches C and D) or pigs from neighbouring

batches, especially in lairages with open fencing.
Since only four batches were included in this study and since lorries and lairages were not tested before they held pigs, no conclusions regarding the relative importance of environmental contamination or transmission between pigs can be drawn. Furthermore, the number of environmental wipes tested from each lorry and lairage might be too low to detect environmental contamination with MRSA. Nevertheless, pigs that are negative at loading are unlikely to be the source of contamination for a lorry.
Lorries and lairages are cleaned and disinfected regularly, but the effectiveness of cleaning and disinfection depends on several variables, such as construction materials, exposure time to disinfectants and organic load (Fosse et al., 2009; Rathgeber, 2009). Due to the method of construction of the lairages (concrete floors with rough surfaces), disinfection is probably insufficient to eliminate all micro-organisms.
Pigs from all batches were in contact with pigs from other farms during the time between loading and stunning. Although pigs from different farms are usually kept in separate sections of lorries and lairages, indirect contact is possible, especially through excreta. Transmission of MRSA between pigs from different farms is therefore a plausible explanation for our findings.
Another, less likely, source of MRSA for pigs in lorries and lairages might have been the lorry drivers and/or abattoir personnel. Studies in pig and poultry abattoirs have demonstrated MRSA prevalences of 5.6% among abattoir workers and 22% among lorry drivers (Mulders et al., 2010; Van Cleef et al., 2010). Transmission of MRSA from humans to animals has been described for dogs and horses (Van Duijkeren et al., 2004b, 2010). However, no information on this route of transmission is available for pigs and lorry drivers, and abattoir personnel were not tested for MRSA in the present study.
The *spa* types identified in this study have all been isolated previously from livestock and assigned to CC398 (Huijsdens et al., 2009). Since t011 and t108 are the most prevalent MRSA types in The Netherlands (Huijsdens et al., 2009), no conclusions about transmission can be drawn on the basis of their consecutive occurrence in pigs and the environment in his study.

CONCLUSIONS

This study demonstrates that negatively tested pigs can become MRSA positive within hours during transportation from the farm to the abattoir. Because of the limited number of batches included in our study, there is a need for the mechanisms of transmission to be elucidated more closely. Nevertheless, the increase in MRSA positive pigs from 0% to 60% in the short time between loading on the farm and stunning at the abattoir indicates very rapid transmission.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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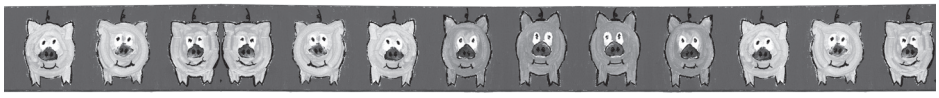
CHAPTER 3.2

HIGH PREVALENCE OF NASAL MRSA CARRIAGE IN SLAUGHTERHOUSE WORKERS IN CONTACT WITH LIVE PIGS IN THE NETHERLANDS



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KEYWORDS

- Abattoirs
- Cross-sectional studies
- Domestic animals
- Humans
- Methicillin resistant *Staphylococcus aureus*

ABSTRACT

Livestock-associated MRSA has been found in various animals, livestock farmers and retail meat. This study aimed to determine the prevalence and determinants of nasal MRSA carriage in pig slaughterhouse workers. Three large pig slaughterhouses in The Netherlands were studied in 2008 using human and environmental samples. The overall prevalence of nasal MRSA carriage in employees of pig slaughterhouses was 5.6% (14/249) (95% CI: 3.4–9.2) and working with live pigs was the single most important factor for being MRSA positive (OR = 38.2; $P < 0.0001$). At the start of the day MRSA was only found in environmental samples from the lairages (10/12), whereas at the end of the day MRSA was found in the lairages (11/12), the dirty (5/12) and clean (3/12) areas and green offal (1/3). The MRSA status of the environmental samples correlated well with the MRSA status of humans working in these sections ($r = 0.75$). In conclusion, a high prevalence of nasal MRSA carriage was found in pig-slaughterhouse workers, and working with live pigs is the most important risk factor. Exact transmission routes from animals to humans remain to be elucidated in order to enable application of targeted preventive measures.

INTRODUCTION

Since 2003, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA), related to the livestock reservoir has emerged in the human population (Voss et al., 2005). As this clone was found to be non-typable (NT) by pulsed-field gel electrophoresis using the *Sma*I restriction enzyme, it was originally called NT-MRSA (Bens et al., 2006; De Neeling et al., 2007). Multi-locus sequence typing revealed that all strains belonged to the clonal complex 398 (CC398) (Huijsdens et al., 2006). At present, it is clear that people who have frequent contact with pigs or veal calves have extremely high MRSA CC398 carriage rates compared to national community prevalences (25–35% vs. 0.1% in The Netherlands) (SWAB, 2008; Graveland et al., 2008; Wertheim et al., 2004a; Van Den Broek et al., 2009). As a result of the elevated prevalences in this specific population, the 'search and destroy' policy in The Netherlands was adapted; persons in contact with live pigs and veal calves are added to the high-risk group and should be screened for MRSA upon hospital admission (WIP, 2007). As a consequence, the number of MRSA CC398-carrying patients found in The Netherlands increased dramatically to nearly 30% of all newly detected MRSA strains in 2007 (Haenen et al., 2009), and 42% in 2008 (SWAB, 2009). The proportion of MRSA in *S. aureus* nosocomial infections remained very low (< 2%), compared to other countries (EARSS, 2007). In a recent survey by the Food and Consumer Product Safety Authority in the Netherlands (VWA) MRSA was found in 11% of retail meat (with a minimum MRSA prevalence of 2% in game and a maximum of 35% in turkey) (De Boer et al., 2009). Other studies also found MRSA in retail meat, in varying percentages (2.5% (Van Loo et al., 2007b), 19% (Lin et al., 2009), 0.7% (Pereira et al., 2009), 5% (Pu et al., 2009), 0% (Lee do et al., 2008) and 17% (De Jonge et al., 2010)).

In animal husbandry-dense areas, the majority of newly identified human MRSA carriers concerns this livestock-associated MRSA (Van Rijen et al., 2008), and recently, the first hospital outbreaks of CC398 have been reported (Wulf et al., 2008a; Fanoy et al., 2009). Meanwhile, serious invasive infections due to CC398 have been observed (Ekkelenkamp et al., 2006; Declercq et al., 2008; Van Loo et al., 2007a; Witte et al., 2007; Lewis et al., 2008; Robicsek et al., 2008). Therefore, the emergence of this new livestock-associated clone poses a potential public health risk that warrants close monitoring.

The high prevalence of MRSA in meat products and in people working with livestock raises the question whether slaughterhouse workers, who are in contact with pigs (dead or alive) and meat products, are also at risk. Therefore, we performed a cross-sectional survey on nasal MRSA CC398 carriage in employees of pig slaughterhouses, and on the occurrence of MRSA in different slaughterhouse sections.

METHODS

Study population, questionnaires and human sampling

Three pig slaughterhouses were enrolled in the study on the basis of voluntary participation, from a complete list of 10 large pig slaughterhouses in The Netherlands. All were located in the south and the east of the country, in areas with a high pig density. By using a structured questionnaire, slaughterhouse-specific information was collected, e.g. number of employees, slaughterhouse capacity, specifics on lairages and the production process, information on microbiological contamination of the carcasses and working benches and hygiene measures.

Slaughterhouse workers were enrolled in the survey based on voluntary participation. A written consent was obtained from each participant. The survey contained questions on age, gender, country of birth, recent antibiotic use, job description, working in more than one section of the slaughterhouse (rotation), wearing plastic gloves, living on a livestock farm, and contact with family members working in healthcare or in livestock farming. Slaughterhouse workers were divided in three different categories according to their activities: contact with live pigs, dead pigs or other. When subjects indicated that they worked in more than one section, they were included in the category with the most intense contact with live animals.

Nasal swabs (Venturi Transystem, Copan Innovation, Italy) were taken from workers in order to determine the presence of MRSA. This study was approved by the Medical Ethical Committee of the University Hospital Utrecht (file no. 08/050).

Environmental sampling

To determine the MRSA status of the different slaughterhouse sections, environmental wipe samples were taken from surfaces in each section (Fig. 1) at the beginning and at the end of the working day using Sodibox wipes (Raisio Diagnostics B.V. Nieuwerkerk aan den IJssel, The Netherlands). Sections of the slaughterhouse were divided in two different categories according to the cleanliness of the animal/carcass: dirty or clean areas. In the dirty area, the carcass surface is cleaned by scalding, depilation and singeing. In the clean area, the carcass is eviscerated and processed into meat products.

Microbiological methods

Nasal swabs were incubated in Mueller Hinton enrichment broth (Becton Dickinson, USA) with 6.5% NaCl, for 18 – 48 h at 35°C. Then 10 µl of the broth was plated onto a MRSA ID culture plate (BioMérieux, France), and incubated overnight at 35°C. Suspect (green) colonies were identified as *S. aureus* by a latex agglutination test (Staphaurex Plus; Murex Diagnostics Ltd, UK) and tested for cefoxitin sensitivity by the disc diffusion method (CLSI, 2007). The obtained MRSA isolates were subsequently stored at –80°C.

Environmental sample wipes were soaked in 100 mL Mueller Hinton enrichment broth with 6·5% NaCl and incubated for 18 h at 37°C. Next, 1 mL of the broth was transferred into 9 mL Phenol Red mannitol broth with 5 mg/mL ceftizoxime and 75 mg/mL aztreonam (BioMérieux) and incubated for 18 h at 37°C. Subsequently, 10

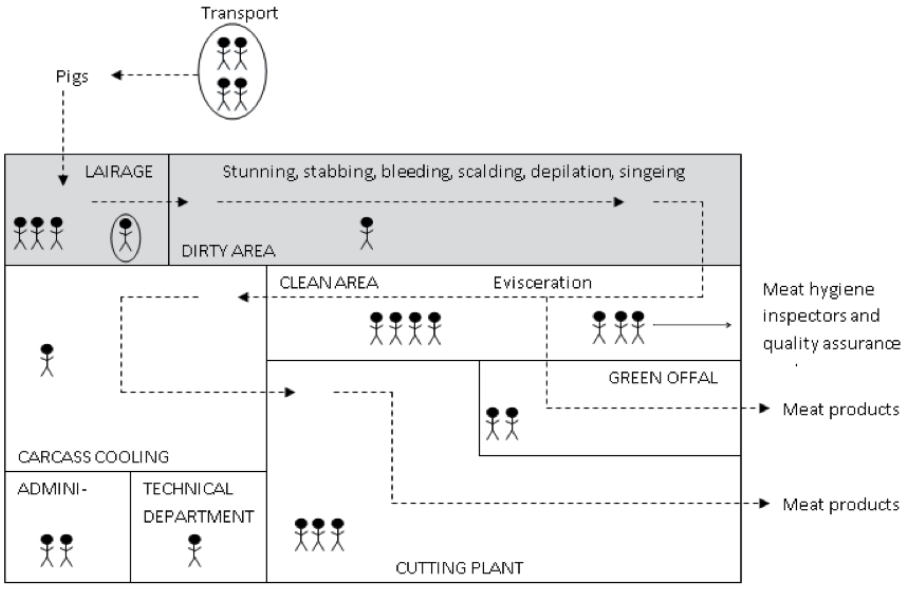


Figure 1. Schematic representation of the sections of the production chain (dotted lines) in a pig slaughterhouse. The shaded area stands for sections where live pigs are located (dirty area). Each human figure represents approximately 10 persons, circled persons do not belong to the actual slaughterhouse employees (livestock transport workers and official veterinarians and auxiliaries).

µL of the suspension was transferred onto a Columbia agar plate with 5% sheep blood. In parallel, Brilliance MRSA culture plates (Oxoid, UK) were inoculated with 10 µL suspension and incubated for 18 h at 37°C. Colonies were subcultured until pure.

Confirmation of the isolates was done by a multiplex PCR specific for *S. aureus* (Martineau et al., 1998), the *mecA* gene (De Neeling et al., 1998) and the Panton–Valentine leucocidin (PVL) toxin genes (Lina et al., 1999). Isolates were defined as MRSA on the basis of their *mecA* gene presence. Staphylococcal protein A *(spa)* typing was conducted according to Harmsen et al. (Harmsen et al., 2003). On all MRSA positive environmental and human samples, antimicrobial susceptibility was tested using the Vitek system (BioMérieux SA, France) according to the manufacturer's instructions.

Sample size and statistical analysis

The prevalence of MRSA nasal carriage in the general population in The Netherlands was assumed to be < 0.5%. A nasal carriage rate of 2% in slaughterhouse workers was considered as a significant increase. The required sample size was calculated as 450 subjects ($\alpha = 0.05$, $\beta = 0.10$).

Prevalence of MRSA in slaughterhouse workers was calculated as a percentage of the total amount of samples in general and specified per category and job descrip-

tion. Wilson confidence intervals (CI) were calculated. Univariable exact logistic regression was performed using SAS, version 9.1 (SAS, 2004). Odds ratios (OR) were determined by comparing different categories and job descriptions within those categories. In order to calculate the association between the human and environmental samples and because of the skewed distributions of the percentages of positive persons and environmental samples per section, Spearman's rank correlation was used.

RESULTS

Slaughterhouse characteristics

In the three selected slaughterhouses, the total number of employees varied between 80 and 260. The total number of slaughtered pigs per day varied between 3800 and 5000, all pigs originated from farms in The Netherlands. In one slaughterhouse, cattle were slaughtered as well, but in separate rooms in the same building.

Humans

Of the total of 497 slaughterhouse workers 195 (39.2%) agreed to participate. An additional 41 livestock transport workers and 13 official veterinarians and auxiliaries (i.e. persons from the VWA, who monitor and assist the meat hygiene inspectors) were included, yielding a total of 249 study subjects, including 16 female participants. Mean age was 43 years (range 19 – 73 years), and the mean working week was 41 h (range 7 – 80 h).

We found an overall nasal MRSA prevalence of 5.6% in slaughterhouse workers (14/249, Table 1). MRSA carriage was found exclusively in persons having contact with live pigs (15.1%), compared to subjects not working with live pigs (0.0%; OR = 38.2, Table 2).

Nine of the 41 (22%) livestock transport workers were MRSA positive, as well as 2/13 (15%) veterinarians and auxiliaries. In total, 3/195 (1.5%; 95% CI: 0.5–4.4) employees of slaughterhouses (excluding livestock transport workers and official veterinarians and auxiliaries) were MRSA positive; these were all working in the dirty area of the slaughterhouse. No specific slaughterhouse function proved to be a significant risk factor, when comparing different activities within the clean and the dirty areas. Twenty-three persons indicated working in both dirty and clean areas and only one of these was found MRSA positive.

Regarding potential determinants and confounders, no significant difference in persons with and without MRSA was found (Table 2). Furthermore, no significant differences in MRSA prevalence in humans between slaughterhouses were found.

Table 1. Prevalence of nasal MRSA carriage in slaughterhouse workers (data from 3 slaughterhouses combined).

Contact with pigs	Function	Total	MRSA	Percentage	95 % CI
Live pigs	Livestock transport worker	41	9	22.0	12.0 - 36.7
	Official veterinarian + auxiliary	13	2	15.4	4.3 - 42.2
	Lairage worker	32	2	6.3	1.7 - 20.1
	Dirty area worker	7	1	14.3	2.6 - 51.3
Dead pigs[a]		127	0	0.0	0.0 - 2.9
Other[b]		29	0	0.0	0.0 - 11.7
Total		249	14	5.6	3.4 - 9.2

[a] Clean area worker, carcass cooling and cutting plant worker, green offal worker, meat hygiene inspector, quality assurance worker.

[b] Administrative and technical personnel.

Table 2. Univariable exact logistic regression analysis.

Characteristic	Total	MRSA	Percentage	OR	95% CI	*P*-value
Female gender	16	0	0.0	Ref.		
Male gender	233	14	6.0	1.4	0.2 - ∞	0.77
Born abroad	60	1	1.7	0.2	0.0 – 1.6	0.22
Living on livestock farm	24	3	12.5	2.8	0.5 – 11.7	0.28
Recent antibiotic use	28	3	10.7	2.3	0.4 – 9.5	0.40
Contact with family members in healthcare or livestock-farming	47	3	6.4	1.2	0.2 – 4.7	1.00
Working with live pigs	**93**	**14**	**15.1**	**38.2**	**6.3 - ∞**	**<0.0001**
Rotation	59	3	5.1	0.9	0.3 – 3.5	1.00
Always wearing plastic gloves	53	2	3.8	Ref.		
Sometimes wearing plastic gloves	76	6	7.9	2.2	0.4 – 22.9	0.57
Never wearing plastic gloves	113	6	5.3	1.4	0.2 – 14.9	1.00

Boldface values belong to characteristics that are significantly related to MRSA, when comparing the presence of the concerning factor vs. the absence of it.

Environment

At the start of the day MRSA was only found in environmental samples from the lairages (10/12) (Table 3, Fig. 1). At the end of the day MRSA was found in the lairages (11/12), the dirty (5/12) and clean (3/12) areas and green offal (1/3). Spearman's correlation coefficient, a measure for the correlation between MRSA status of the environmental samples and the humans working in these areas, is 0.75 (P = 0.002). The squared correlation (0.75 × 0.75 = 0.56) gives the coefficient of determination; 56% of variance in percentage of positive persons can be explained by environmental contamination.

Table 3. MRSA in environmental samples taken at start and end of working day (data from 3 slaughterhouses combined).

Pigs	Department	Start of the day			End of the day		
		Total	MRSA	Percentage	Total	MRSA	Percentage
Live	Lairage	12	10	83.3	12	11	91.7
	Dirty area	12	0	0.0	12	5	41.7
Dead	Clean area	12	0	0.0	12	3	25.0
	Carcass cooling	12	0	0.0	12	0	0.0
	Cutting plant	8	0	0.0	8	0	0.0
	Green offal	3	0	0.0	3	1	30.0

Spa typing and antimicrobial susceptibility testing

In total, 14 human and 32 environmental MRSA strains were collected. The predominant *spa* type was t011 in both human subjects (11/14) and environmental samples (21/32). *Spa* type t108 was only found once in a human nasal sample, and also once in an environmental sample from the corresponding slaughterhouse. An additional 10 environmental isolates from the other slaughterhouses were typed as t108. *Spa* type t571 was only found once in environmental samples, and t034 and t1451 were found only once in humans, not in environmental samples of the corresponding slaughterhouse. From two environmental samples two different *spa* types were isolated, in both cases t011 and t108. PVL-positive strains were not found.

Antimicrobial susceptibility testing revealed that all MRSA isolates from humans and the environment are resistant against tetracycline (Table 4), and 19/46 isolates show combined erythromycin and clindamycin resistance. Furthermore, all isolates are sensitive for mupirocin and vancomycin (only human isolates tested). *Spa* type t108 appears to have less combined erythromycin and clindamycin resistance (0/11 = 0.0%) than t011 (17/32 = 53.1%; P = 0.002). No clear difference in resistance pattern between the human and environmental isolates was determined.

Table 4. Antimicrobial susceptibility profiles of all human and environmental MRSA isolates.

Antimicrobial	Human (*n*=14)		Environmental (*n*=32)	
	Resistant	Percentage	Resistant	Percentage
Tetracycline	14	100.0	32	100.0
Erythromycin	8	57.1	12	37.5
Clindamycin	8	57.1	12	37.5
Gentamicin	1	7.1	11	34.4
Ciprofloxacin	0	0.0	6	18.8
Trimethoprim/sulfamethoxazole	3	21.4	1	3.1
Rifampicin	0	0.0	0	0.0
Fusidic acid	0	0.0	0	0.0
Linezolid	0	0.0	0	0.0
Mupirocin	0	0.0	0	0.0
Tobramycin	1	7.1	n.t.	
Vancomycin	0	0.0	n.t.	
Nitrofurantoin	0	0.0	n.t.	
Neomycin	n.t.		1	3.1
Amikacin	n.t.		0	0.0

n.t. = not tested.

DISCUSSION

To our knowledge, this is the first study on the prevalence of nasal MRSA in pig slaughterhouse workers. Working with live pigs is the most important determinant for nasal CC398 carriage, justifying the present hospital infection control guidelines in The Netherlands, which indicate that contact with live pigs is a risk factor for MRSA carriage. Working with dead pigs does not seem to be a risk factor for MRSA carriage.

The prevalence of 15.1% in persons working with live pigs is comparable to data found elsewhere, e.g. 26% and 14% in pig farmers and 12.5% in veterinarians attending an international pig health convention (Voss et al., 2005; Wulf et al., 2008b; Van Den Broek et al., 2009). A low prevalence was found in Danish veterinarians (3.9%) (Moodley et al., 2008), but higher nasal prevalences were found in German pig farmers on MRSA positive farms (86%), German pig veterinarians (45%) and USA pig farmers (45%) (Cuny et al., 2009; Smith et al., 2009).

The overall MRSA prevalence in all subjects in the current study is 5.6%, which is significantly higher than the general population prevalence reported in

The Netherlands (0.1%) (Wertheim et al., 2004a; Donker and Stobberingh, 2008; SWAB, 2008). The higher prevalence in livestock transport workers compared to lairage workers might be explained by the less intense physical contact with pigs by lairage workers, who often use sticks to herd the animals. Transport workers earmark all animals at pick up and often herd the animals with their bare hands. Second, high-pressure spray cleaning of the truck may result in formation of MRSA aerosols, which can be inhaled by the transport worker. Insight into these mechanisms may give more information on the transmission route of MRSA.

During the day MRSA accumulates, particularly in the first stages of the production process, which predominantly deals with live pigs. Since pigs were loaded into the lairages at night, the lairages were not clean at the time of sample collection at the beginning of the day. Moreover, the lairages are cleaned every day, but not disinfected.

There is a significant association between the presence of MRSA in different sections, and the percentage of MRSA positive persons working in these relevant sections. It is possible that acquisition of MRSA occurs through contaminated surfaces (Boyce, 2007). However, presence of MRSA on different surfaces does not necessarily imply that there is an increased risk of human MRSA acquisition via the environment: where the lairages have a high percentage of MRSA positive samples at the end of the day (92%), a relatively low percentage of lairage workers had acquired the bacterium (6.3%). It is plausible that animals spread MRSA to both humans and the environment, and human acquisition of MRSA seems to be more likely by contact with MRSA positive animals than through environments with MRSA in dust or aerosols.

All *spa* types found in our study were previously confirmed as belonging to the CC398 livestock-associated MRSA clone (Huijsdens et al., 2009). The most predominant *spa* types in both human and environmental isolates were t011 and t108, which is in accord with previous studies in pigs and pig farmers (Armand-Lefevre et al., 2005; Voss et al., 2005; Huijsdens et al., 2006; Van Loo et al., 2007a; Van Belkum et al., 2008; Van Den Broek et al., 2009). The subject with t034 was an official veterinarian and the *spa* type t1451 came from a livestock transport worker, these persons often have more animal contacts than in the slaughterhouse alone. Antimicrobial susceptibility, in particular tetracycline resistance was comparable to profiles found in other studies for livestock-associated MRSA (De Neeling et al., 2007; Van Loo et al., 2007a; Van Den Broek et al., 2009).

The prevalence of MRSA found in retail meat in other studies is considerable; the prevalence of MRSA found in employees of pig slaughterhouses in this study is low. The role of slaughterhouse employees in transmitting MRSA to the meat products thus does not seem to be large. Especially as persons working with meat products were all negative in this study. This finding is in accord with an unpublished study (De Jonge et al., 2010), where none of 101 employees from the cold-meat processing industry and institutional kitchens carried MRSA. It is probable that another transmission route to retail meat is involved here. Contamination of meat with MRSA by the environment (surfaces) and/or equipment, or from animals to carcasses/meat products is more likely to occur.

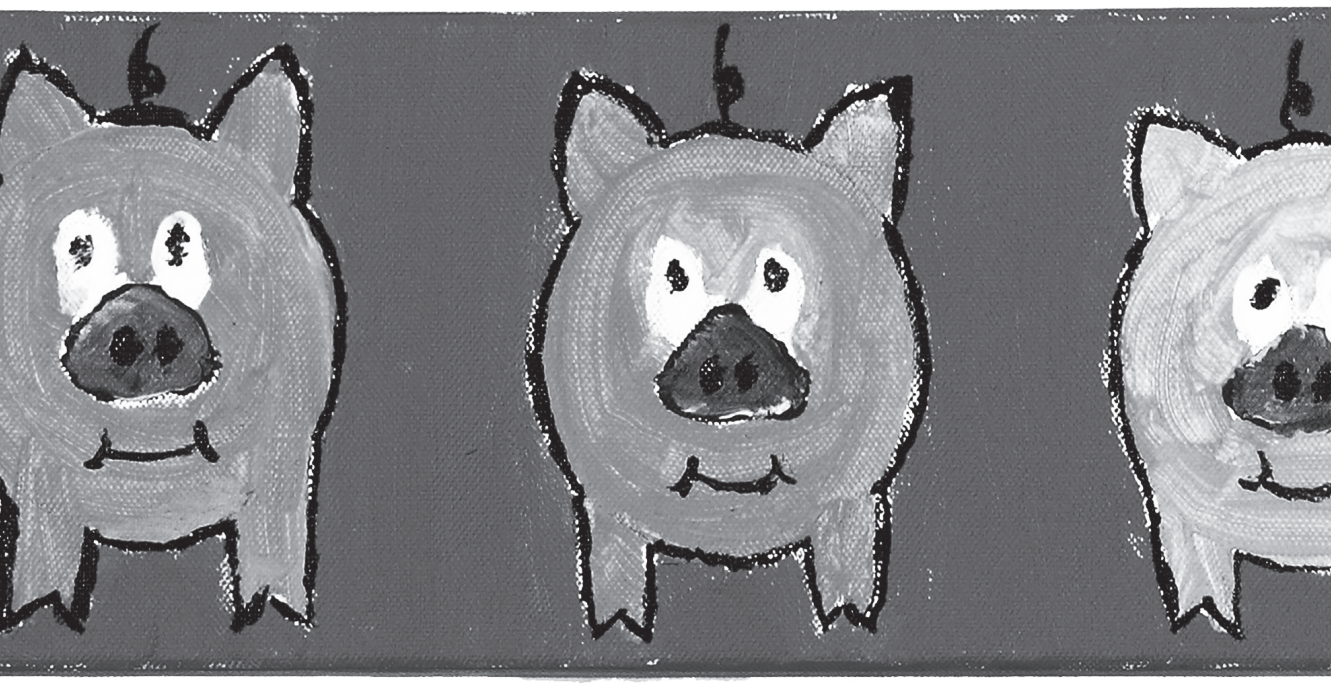
This kind of cross-contamination has already been demonstrated for *Salmonella* spp. in pig slaughterhouses (Prendergast et al., 2008).

Our study has a few limitations. As with every questionnaire, survey recall bias, selection bias, and language bias may have occurred. Next, the low number of slaughterhouses visited (n = 3) yields little power to find significant differences between slaughterhouses. Nevertheless, we assume that these results are representative for all Dutch pig slaughterhouses, because the working conditions in all pig slaughterhouses in The Netherlands are comparable due to automation and the strict legislation on hygiene and animal handling. Despite a smaller sample size than calculated beforehand, the number of subjects is still sufficient to confirm previous findings on the risk of acquiring MRSA for people in contact with live pigs. Possibly more risk factors could be found if the number of slaughterhouse workers was larger, e.g. country of birth, recent antibiotic use, amount of hours worked per week, and contact with healthcare. Furthermore, no pigs were sampled in our study, but in a previous study on MRSA at Dutch slaughterhouses MRSA was detected in 81% of the Dutch slaughter batches and 39% of the individual pigs (De Neeling et al., 2007). Environmental samples are considered to be a good proxy for animal MRSA carriage, concerning the association found between environmental and animal samples in other studies (OR 27.5; $\kappa = 0.68$) (Broens et al., 2008a). Longitudinal information on duration of MRSA carriage and the possibility of transient colonization is not yet available; this will be our group's next study subject.

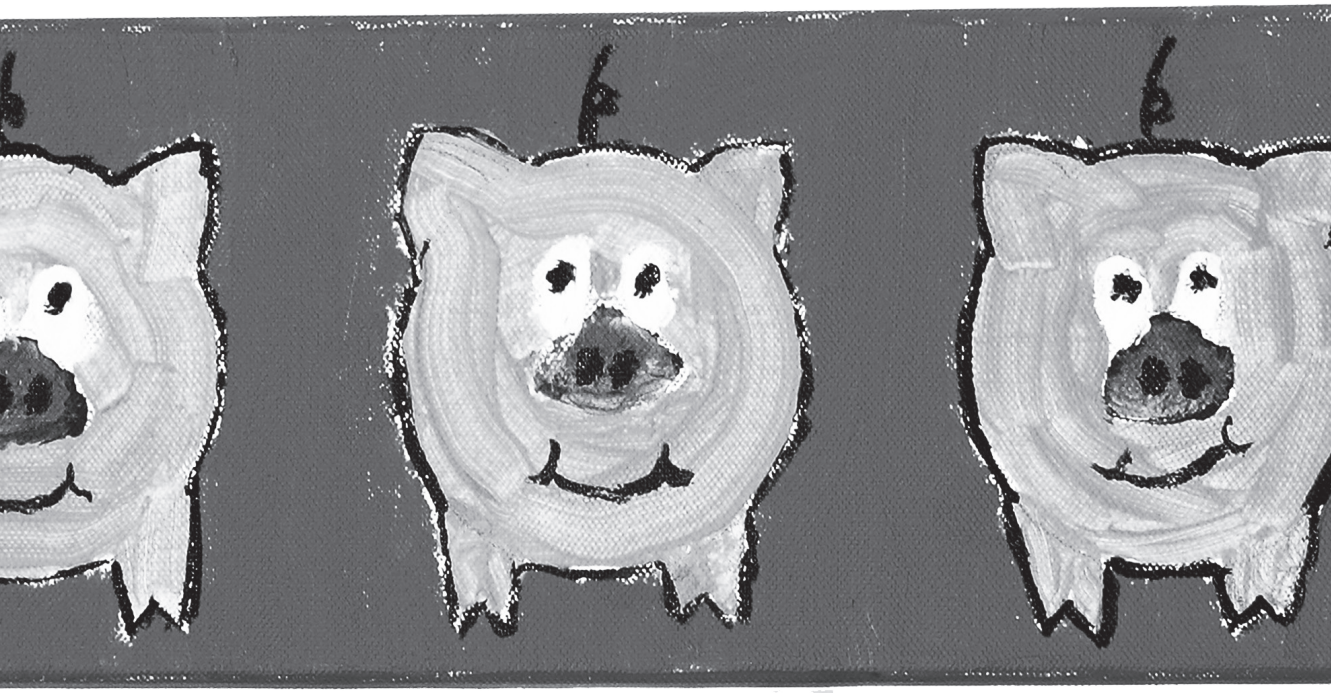
In conclusion, nasal MRSA CC398 is found in pig slaughterhouse workers in significantly higher percentages than the general population prevalence in The Netherlands. It is found exclusively in persons working with live pigs. In addition to contact with live pigs, environmental contamination might also play a role in the acquisition of MRSA, but exact transmission routes from animals to humans remain to be elucidated in order to enable application of targeted preventive measures.

Acknowledgements

We thank the three slaughterhouses and in particular their quality assurance workers for their participation and help in the data collection in this study.

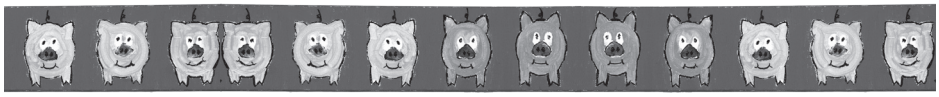


PART IV – TRANSMISSION DYNAMICS OF LA-MRSA IN PIG POPULATIONS



CHAPTER 4.1

QUANTIFICATION OF TRANSMISSION OF LIVESTOCK-ASSOCIATED METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN PIGS



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KEYWORDS

- Methicillin resistant
- *Staphylococcus aureus*
- Pigs
- Transmission
- Colonization
- Reproduction ratio
- MRSA

ABSTRACT

Antimicrobial resistance in pigs becomes a public health issue when resistant organisms transfer from pigs to humans. Pigs are a large reservoir for livestock-associated (LA-)MRSA and people in contact with pigs are at risk for infection with LA-MRSA. Transmission and persistence of LA-MRSA within a pig population contributes to the maintenance of this zoonotic reservoir. Current knowledge on colonization and transmission of LA-MRSA in pigs is limited and mainly based on observational field surveys. Two experiments were performed to colonize pigs and quantify transmission of LA-MRSA between pigs. In the first experiment, colonization of six-week old piglets failed after intranasal inoculation, confirming the complexity of MRSA colonization. In the second experiment, naïve pigs got colonized after exposure to orally inoculated pigs. Subsequently, these contact-infected pigs transmitted MRSA to a new group of naïve pigs. The reproduction ratio, R_0, was estimated with a *SIS*-model to quantify transmission between the first and second contact pigs as this resembles more the natural transmission. Two scenarios were evaluated, with different assumptions regarding infection status of individual pigs. R_0 varied between 3.7 and 4.3 and was significantly above 1, indicating a high probability of persistence of LA-MRSA, even without antimicrobial use.

INTRODUCTION

In 2004, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA), referred to as livestock-associated (LA-)MRSA, was found in pigs and in people in contact with pigs (Voss et al., 2005). Since then, many countries have detected LA-MRSA in pigs, and in people in contact with pigs (Broens et al., 2008c; EFSA, 2009; Smith et al., 2009; Van Den Broek et al., 2009). Pigs are therefore, a zoonotic reservoir of MRSA for humans. Surveys on Dutch farms and slaughterhouses identified contact of humans with positive livestock as an important risk factor for human MRSA carriage, where the intensity of contact was strongly associated with increased prevalence (Van Den Broek et al., 2009; Graveland et al., 2010; Mulders et al., 2010; Van Cleef et al., 2010).

In humans, carriers of *S. aureus*, including MRSA, could be persistent or intermittent carriers. Host factors seem to influence the individual susceptibility to colonization (Van Belkum et al., 2009). The primary route of MRSA transmission between humans seems to be direct contact with individuals carrying MRSA (Eveillard et al., 2004; Albrich and Harbarth, 2008). However, environmental spread might be a substantially underestimated route for MRSA transmission in hospitals (Sexton et al., 2006; Boyce, 2007). Data on MRSA colonization and –transmission in and between individual pigs are limited so far. Current knowledge on transmission of LA-MRSA mostly concerns transmission between herds and is mainly based on observational field surveys (Broens et al., 2010, 2011b). The presence of LA-MRSA in environmental samples taken on pig farms (Broens et al., 2011a), might indicate that direct contact with pigs is not necessarily needed for transmission between pigs and between pigs and human.

At least 2 requirements within a pig population are needed to enable transmission of LA-MRSA from pigs to humans: (1) LA-MRSA should be transmitted between pigs and (2) LA-MRSA should be able to persist in a pig population for a longer time. Experimental studies have proven to be useful in understanding transmission of bacterial infections by quantification of the basic reproduction ratio (R_0), as a measure of transmission (Velthuis et al., 2003; Geenen et al., 2005; Velthuis et al., 2007). The basic reproduction ratio is the average number of secondary cases caused by one typical infectious individual during its entire infectious period in a completely susceptible population (Kermack and McKendrick, 1991; De Jong and Kimman, 1994). R_0 has a threshold value of 1; if $R_0 > 1$, minor and major outbreaks can occur, and an endemic equilibrium can be established and maintained, whereas if $R_0 < 1$ an infection will fade out (De Jong and Kimman, 1994; Velthuis et al., 2007).

Two experiments were performed to meet the following objectives: (1) to establish a method for colonization of pigs with LA-MRSA and (2) to quantify the reproduction ratio for LA-MRSA between pigs.

MATERIALS AND METHODS

Colonization experiment

Five conventionally reared, six-week old female pigs from a confirmed MRSA negative farm were used in the experiment. A week before the start of the experiment (day -7), the pigs were transferred to the experimental animal facilities at the Central Veterinary Institute (Lelystad, the Netherlands). All pigs were individually housed in separate units. To standardize the residential nasal flora, all pigs were intranasal inoculated using a syringe with 1 mL inoculum per nostril containing 10^8 CFU/mL of a methicillin-sensitive *Staphylococcus aureus* (MSSA) strain at day 0. This MSSA strain, with *spa* type t337, was isolated from pigs in a previous study on pig farms. Two weeks after MSSA inoculation (day 14), all pigs were intranasal inoculated using a syringe with 1 mL inoculum per nostril containing 10^8 CFU/mL of a LA-MRSA strain. This strain, with *spa* type t011, was isolated from pigs in a previous study on pig farms. The experiment was terminated at day 55.

Transmission experiment

Due to unsatisfactory results of the colonization experiment, a second experiment was performed which differed in, e.g. housing of pigs and inoculation method. The design of this transmission experiment is a modification of the extended transmission experiment described by Velthuis et al. for *Actinobacillus pleuropneumoniae* (2003).

Fifteen conventionally reared, six-week-old castrated male pigs from a confirmed MRSA negative farm were used. A week before the inoculation (day -7), the pigs were transferred to the experimental animal facilities at the Central Veterinary Institute (Lelystad, the Netherlands) and randomly assigned to three different groups (C_0 = inoculated pigs; C_1 = first contact pigs; C_2 = second contact pigs). The C_0- and C_1-group were housed together, whereas the C_2-group was housed in a separate unit. On day 0 (= day of inoculation) the C_1-group was separated temporarily from the C_0-group during inoculation. To start the infection chain, the C_0-group was orally inoculated using a syringe with 50mL inoculum containing 10^8 CFU/mL of a LA-MRSA strain, *spa* type t011, the same strain as used in the colonization experiment. Several hours after inoculation, the C_1-group was reunited with the C_0-group. When C_1-pigs confirmed to be MRSA positive, the C_0-group was removed and euthanized. The C_1-group was then placed together with the C_2-group, and these groups remained together till day 62, when the experiment was terminated.

Both experiments were approved by the Animal Experimentation Board of Wageningen University.

Sample collection and processing

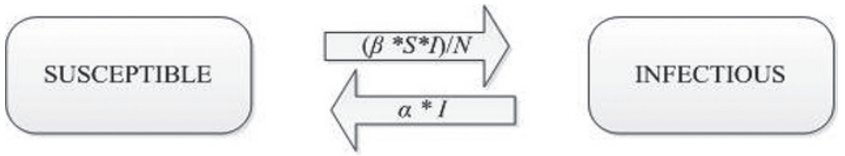
In the colonization experiment, nasal, rectal and vaginal swabs (Medical Wire and Equipment, MW102, United Kingdom) were taken twice a week. In the transmission experiment, nasal and rectal samples were taken twice a week. Samples were stored at 4°C, transported to the lab and analysed within seven days after

sampling. All individual swabs were put into Mueller Hinton Broth with 6.5% NaCl (MHB+) and cultured for MSSA and MRSA. In the colonization experiment MSSA culture was done for all samples; in the transmission experiment this was limited to the swabs taken at arrival and at the end of the experiment. For MSSA isolation, a loop-full (10 µL) of MHB+ was spread onto Sa Select agar (Biorad, 63748, France) and one suspected colony per plate was then confirmed to be *S. aureus* using standard techniques, i.e. colony morphology and slide coagulase test (Graveland et al., 2009). For MRSA isolation, 1 mL of MHB+ was transferred into 9 mL of Phenol Red Mannitol Broth with 4 mg/L ceftizoxime and 75mg/L aztreonam (BioMérieux, NL020, France) and cultured for 18h at 37°C. Subsequently, a loop-full (10 µL) of PMB+ was spread onto a chromogenic MRSA screen agar (Oxoid, PO5196A, United Kingdom). One suspected colony per plate was then confirmed to be MRSA by PCR for the *mecA* gene (De Neeling et al., 1998). All confirmed MSSA and MRSA isolates were stored at -80 °C. *Spa* typing (Harmsen et al., 2003) was done on all isolates from the colonization experiment and on a selection of isolates from the transmission experiment. From the latter experiment, all isolates from swabs taken on day -7, day 15 and day 62 were selected for *spa* typing. Post-mortem examination was performed on pigs that died during the transmission experiment. Affected tissues were collected for further microscopic and microbiological examination. At the end of the transmission experiment, all C_1- and C_2-pigs were euthanized and macroscopic post-mortem examination was performed.

Statistical analysis

Pigs were classified MSSA or MRSA positive if either one of the swabs (nasal, rectal or vaginal) tested positive for MSSA or MRSA, respectively. To estimate transmission parameters it is essential to determine the infection status of individual pigs at each sampling moment. In the transmission experiment, two scenarios regarding microbiological test results of the pigs at each sampling moment were applied. Scenario (1): a pig was classified infectious *(I)* when tested positive in any of the samples or susceptible *(S)* when tested negative in all samples; scenario (2): a pig was classified infectious *(I)* when tested positive in any of the samples at two consecutive samplings or susceptible *(S)* when tested negative in all samples at two consecutive samplings.

Based on our bacteriological results and on the fact that most humans are intermittent carriers (Van Belkum et al., 2009), it was assumed that infectious pigs stop shedding after a while and become susceptible again. Therefore, a susceptible-infectious-susceptible *(SIS)* model (Velthuis et al., 2007) was used to describe the transmission of MRSA in the experiment. The model can be represented as follows:



The number of infection events in a population with size *N (= S + I)* depends on the number of susceptible *(S)*, the number of infectious *(I)* individuals, the total number of animals *(N)*, and the transmission parameter β. Recovery events depend on the number of infectious *(I)* individuals and the recovery parameter α. The transmission parameter β, i.e. the number of secondary cases *(C)* out of a number of susceptible individuals *(S)* caused by infectious individuals *(I)* during each time interval *(Δt)*, was estimated using a function of *I, S, C, N* and Δt. Underlying assumptions were, that (1) all individuals were randomly in contact, (2) *S*- and *I*-individuals were homogeneous groups, i.e. all individuals were equally susceptible or infectious, and (3) all infectious individuals were equally infectious over time, i.e. constant infection rate during the entire infectious period. Data were statistically analysed with SAS version 9.1 software using Generalized Linear Models (PROC GENMOD) with a complementary log-log link function, the term log *((I/N)*Δt)* as offset, *C* as the number of new cases, and *S* as the number of trials in the binomial process (SAS, 2004). Because the probability *(p)* to become infected in the binomial model can be described as: $p = 1 - e^{-\beta (I/N)\Delta t}$, the linear relationship for the statistical model, i.e. the relation between the expected value and the offset and explanatory variable (in this case the intercept of the model) is:

$$c \log \log (C/S) = \log \beta + \log (I/N)*\Delta t) \qquad \text{(Velthuis et al., 2003)}$$

Exponentiation of the estimated parameter '$\log \beta$' gives the transmission parameter β. R_0 can then be calculated by multiplying β with the length of the infectious period (1/ α), which was defined as the average number of days that pigs were classified as infectious in the experiment according to the two scenarios. As oral inoculation with a large volume of MRSA suspension does not mimic a natural infection route, only data from C_1- and C_2-pigs were used for transmission quantification.

RESULTS

Colonization experiment

Four of the five pigs tested MSSA positive on the day of arrival. All pigs tested MRSA negative on the day of arrival, and again six days later. All pigs were intermittently MSSA positive throughout the experiment (Table 1). Pig I tested MRSA positive once and pig V tested MRSA positive four consecutive samplings; both after inoculation. Considering these unsatisfactory results, we decided to place all five pigs together at day 45 to enable MRSA transmission between the pigs. No MRSA was isolated after that until the end of the experiment (day 55).

All pigs were sampled 22 times, resulting in 110 samplings in total. In 45.5% (50/110) of the samplings, swabs from all three sampling sites (nasal, rectal, vaginal) tested MSSA negative; in the remaining samplings ($n = 60$), at least one swab per pig tested MSSA positive. In 45 samplings (40.9%), a positive classification was based on a single positive swab (36 nasal, 4 rectal, 5 vaginal). In 10 samplings (9.1%), a positive classification was based on two positive swabs (8 nasal/vaginal, 1 nasal/rectal, 1 rectal/vaginal). In the remaining 5 samplings (4.5%), all

sampling sites tested MSSA positive.
Two *spa* types were detected in the MSSA isolates, i.e. t127 (*n* = 73) and t337 (*n* = 5). All pigs were intermittently colonized with MSSA *spa* type t127 throughout the experiment. MSSA *spa* type t337, which was used to inoculate the pigs at day 0, was isolated from pig I (*n* = 2) and pig III (*n* = 1) soon after inoculation (< 10 days). At the end of the experiment, MSSA *spa* type t337 was isolated from pig II and pig III once. In both pigs, MSSA *spa* type t127 was simultaneously isolated from other swabs taken that day. All MRSA isolates (*n* = 5) belonged to *spa* type t011 (Table 1).

Table 1. MSSA and MRSA classification[a] of individually housed pigs (I-V) at each sampling moment in the colonization experiment

		Days in experiment																								
	Id pig	-7	-1	0	1	3	6	8	10	13	14	15	17	20	22	24	27	30	34	37	41	42	44	48	51	55
MSSA	I	0	1[b]	Inoculation with MSSA t337	0	1[b]	1[b]	1[c]	1[c]	1[b]	Inoculation with MRSA t011	0	1[b]	0	1[b]	1[b]	0	1[b]	0	0	1[b]	All pigs placed together	0	1[b]	0	1[b]
	II	1[b]	1[b]		0	0	0	0	1[b]	0		0	0	0	0	0	0	0	0	0	1[b]		1[b]	1[d]	0	1[b]
	III	1[b]	1[b]		0	0	1[b]	1[c]	0	1[b]		0	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	0	1[b]	1[b]		1[b]	1[b]	0	1[d]
	IV	1[b]	0		0	1[b]	1[b]	1[b]	0	1[b]		1[b]	0	0	0	1[b]	1[b]	0	0	0	0		1[b]	1[b]	0	1[b]
	V	1[b]	1[b]		0	0	1[b]	0	0	1[b]		0	0	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	0	1[b]		0	1[b]	0	1[b]
MRSA	I	0	0		0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	1[d]		0	0	0	0
	II	0	0		0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0
	III	0	0		0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0
	IV	0	0		0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0		0	0	0	0
	V	0	0		0	0	0	0	0	0		0	0	1[e]	1[e]	1[e]	1[e]	0	0	0	0		0	0	0	0

[a] Pigs were classified positive if at least one of the swabs (nasal, rectal, vaginal) tested positive; [b] MSSA *spa* type t127; [c] MSSA *spa* type t337; [d] MSSA *spa* type t337 and t127 in same pig; [e] MRSA *spa* type t011

Transmission experiment

Eleven out of 15 pigs tested MSSA positive on the day of arrival. All 15 pigs tested MRSA negative on the day of arrival, and again six days later. Four out of five C_0-pigs died within 24 hours after inoculation, due to pneumonia (see post-mortem examination). After housing the solely left C_0-pig and C_1-pigs together, all pigs tested MRSA positive at day 2. One C_1-pig died at day 14 without showing any clinical signs prior to death. The remaining C_0- and C_1-pigs stayed MRSA positive until day 15. On that day the C_0-pig was removed and euthanized and the four C_1-pigs were placed together with the five MRSA negative C_2-pigs, confirmed to be MRSA negative at day 15. MRSA was transmitted to all five C_2-pigs, and all pigs became intermittently colonized until the end of the experiment (Table 2).
Pigs were sampled until day 62, resulting in 181 samplings in total. In 45.9%

(83/181) of the samplings, swabs from both sampling sites (nasal, rectal) tested MRSA negative; in the remaining samplings (n = 98), at least one swab per pig tested MRSA positive. In 78 samplings (43.1%), a positive classification was based on a single positive swab (76 nasal, 2 rectal). In the remaining 20 samplings (11.0%), both sampling sites tested MRSA positive.

In total, 36 MSSA isolates and 11 MRSA isolates were *spa* typed. Two *spa* types were detected in the MSSA isolates, i.e. *spa* type t337 (n = 26) and *spa* type t127 (n = 10). Both were found at arrival and at the end of the experiment, either in nasal and rectal swabs, sometimes within the same pig (Table 2). All MRSA isolates belonged to *spa* type t011, which was used to inoculate the C_0-pigs.

Table 2. MRSA classificationa of individual pigs per group at each sampling moment in the transmission experiment

			Days in experiment																				
Id pig	Group	-7	0	2	6	8	13	15	15	21	23	27	30	34	37	41	44	48	51	55	58	62
I	C_0	0[b]	Inoculation C_0-pigs with MRSA t011; C_0- and C_1-pigs together	1	1	1	1	C_1- and C_2-pigs together; C_0-pig removed														
II	C_0	0[d]		†																		
III	C_0	0		†																		
IV	C_0	0[b]		†																		
V	C_0	0[b]		†																		
VI	C_1	0		1	1	1	1		†													
VII	C_1	0[d]		1	1	1	1		1	0	1	0	0	0	0	1	1	1	0	1	1	1[b]
VIII	C_1	0[d]		1	1	1	1		1	0	0	0	0	1	1	1	1	1	0	1	1	0[b]
IX	C_1	0[d]		1	1	1	1		1	1	0	0	0	1	0	1	1	1	1	1	1	1[d]
X	C_1	0[b]		1	1	1	1		1	0	1	1	0	0	0	1	1	1	0	0	0	1[d]
XI	C_2	0[b]							0	1	1	0	0	0	1	1	1	1	1	0	0	0[d]
XII	C_2	0							0	1	0	0	0	0	1	1	1	1	1	1	1	0[b]
XIII	C_2	0[b]							0	1	1	0	1	0	0	1	1	1	0	1	1	1[b]
XIV	C_2	0[b]							0	0	0	0	0	0	1	1	1	1	1	1	1	1[d]
XV	C_2	0[c]							0	0	0	0	0	0	1	1	1	1	1	1	1	1[d]

[a] Pigs were classified positive if at least one of the swabs (nasal, rectal, vaginal) tested positive;
[b] MSSA *spa* type t337; [c] MSSA spa type t127; [d] MSSA *spa* type t337 and t127; all MRSA *spa* type t011;
† died, no sample.

Quantification of MRSA transmission

Number of susceptibles *(S)*, infectious *(I)*, and new cases *(C)* per time interval *(Δt)* were counted for both scenarios and used as model input (Table 3). For scenario 1,

the transmission parameter β was estimated to be 0.42 (95% CI: 0.25-0.66). The average length of the infectious period in this scenario was 10.3 days (SD = 7.7; median = 7.5; min-max: 1-26; n = 24), resulting in a R_0 of 4.30, significantly above 1 (95% CI: 2.60-6.74). For scenario 2, the transmission parameter β was estimated to be 0.21 (95% CI: 0.12-0.38). The average length of the infectious period in this scenario was 17.4 days (SD = 7.9; median = 18.0; min-max: 6-29; n = 15), resulting in a R_0 of 3.66, significantly above 1 (95% CI: 2.02-6.63).

Post-mortem examination

All four C_0-pigs who died within 24 hours after inoculation had necrotizing pneumonia, caused by MRSA. The C_1-pig that died at day 14 had sepsis caused by a severe fibrinopurulent meningoencephalitis; bacteriological examination revealed no pathogenic organism. Macroscopic examination of the nine pigs euthanized at the end of the experiment revealed no visible abnormalities.

Table 3. Input for the transmission model

Day			Scenario 1[a]			Scenario 2[b]		
Start	End	Δt	S	I	C	S	I	C
15	21	6	5	4	3	5	4	2
21	23	2	5	4	2	4	5	0
23	27	4	5	4	0	5	4	0
27	30	3	8	1	1	7	2	0
30	34	4	8	1	2	8	1	1
34	37	3	7	2	4	8	1	4
37	41	4	4	5	4	4	5	4
41	44	3	0	9	0	0	9	0
44	48	4	0	9	0	0	9	0
48	51	3	0	9	0	0	9	0
51	55	4	4	5	3	1	8	0
55	58	3	2	7	0	2	7	0
58	62	4	2	7	1	2	7	0

[a] A pig was classified infectious *(I)* when tested positive or susceptible *(S)* when tested negative.

[b] A pig was classified infectious *(I)* when tested positive at two consecutive samplings or susceptible *(S)* when tested negative at two consecutive samplings.

Δt = time interval (days); S = number of susceptibles at start of time interval; I = number of infectious individuals at start of time interval; C = number of new cases during time interval; population size *(N)* was constant, i.e. 9, for all time intervals.

DISCUSSION

The objective of this paper was to colonize pigs with LA-MRSA and, subsequently, to quantify transmission of LA-MRSA between pigs. Direct intranasal inoculation of individual piglets did not result in colonization with LA-MRSA. The already present MSSA in the nasal flora might have out competed LA-MRSA (Dall'Antonia et al., 2005). Also bacteria of other species or genera might have interfered with colonization (Lina et al., 2003; Frank et al., 2010). Bacterial interference might explain the failure of our inoculation method. Our piglets were already colonized with MSSA *spa* type t127 at arrival and we inoculated them with MSSA *spa* type t337, in an attempt to standardize the residential nasal flora. Both MSSA strains might have interfered with each other and with the MRSA strain we used for inoculation. Since we only *spa* typed one isolate per sample, it is hard to draw any conclusions on frequency and interference between different *spa* types. In a study of Moodley et al. (2011a), piglets were inoculated intranasal and intragastrical with a mixture of four MRSA strains during tetracycline treatment. This inoculation procedure failed to result in MRSA colonization, whereas intravaginal inoculation of sows with the same MRSA mixture resulted in colonization in newborn piglets with two different *spa* types (Moodley et al., 2011a). The mechanisms leading to MRSA colonization seems to be multifactorial: bacterial, environmental and host factors are involved (Van Belkum et al., 2009).

Due to unsatisfactory results in the colonization experiment, we concluded that the intranasal inoculation method was not suitable to start the infection chain of MRSA in pigs. Therefore, we have chosen a larger inoculation volume and an oral inoculation route in the second experiment. This procedure resulted in MRSA transmission and a successful MRSA colonization for over four weeks in contact-infected pigs. However, four out of five inoculated pigs died due to severe pneumonia shortly after the inoculation, which makes this inoculation method not suitable for future experiments. Intragastrical inoculation using an endogastric tube might be a safe and useful procedure, however in the experiment performed by Moodley et al. (2011a) this method did not result in colonization.

After establishment of MRSA colonization in our transmission experiment, MRSA transmission between contact-infected and naïve pigs was studied. Transmission parameters were estimated using a *SIS*-model, which is an endemic model, assuming that infection confers no immunity. Our bacteriological results confirm this assumption as several individuals are intermittently tested MRSA positive, i.e. in terms of our model, susceptibles become infectious, eliminate the bacteria and then become susceptible again. The estimate of the transmission parameter β is highly dependent on the definition of the infection status, either susceptible or infectious, of individual pigs. In a scenario with the assumption that a pig was infectious when tested positive or susceptible when tested negative, β was two times larger than in a scenario with the assumption that a pig was infectious when tested positive at two consecutive samplings or susceptible when tested negative at two consecutive samplings. However, values of R_0 in both scenarios

were similar, due to a much longer observed infectious period (i.e. 17.4 versus 10.3 days) in the second scenario.

We assumed that infectious pigs become susceptible again after a period of shedding, meaning that no final size situation is reached at the end of the experiment. The statistical model we used to estimate transmission parameters does not rely on the final size assumption (Velthuis et al., 2007) and is therefore an appropriate method for analysis of data from this experiment. Other assumptions we made, e.g. randomly contact between pigs and a constant infection and recovery rate, could not be tested in our study. Therefore, the estimate of transmission parameters should be considered a valid estimate of MRSA transmission between pigs.

The reproduction ratio was significantly larger than 1 in both scenarios, indicating a high probability of establishing an endemic equilibrium and, consequently, persistence within a pig population. Based on the estimated R_0-values, the expected proportion of infectious individuals at an endemic equilibrium (Allen and Burgin, 2000) is 77% and 73% for scenario 1 and 2, respectively. Although we cannot tell whether an equilibrium was reached at the end of our experiment, indeed six out of nine pigs (67%) tested MRSA positive at that time. Estimated values of R_0 are applicable for pigs in direct contact (within a pen). Whether LA-MRSA will eventually fade out or persist in a pig population, depends on the number of susceptibles introduced, either by birth or by purchase of pigs, on the effective population size and on the transmission rate between pens (Allen and Burgin, 2000).

Direct contact between MRSA positive and MRSA negative pigs and environmental spread are the most likely routes for MRSA transmission between pigs. Faecal-oral transmission was shown to be a possible route in a murine nasal colonization model (Bloemendaal et al., 2011). Our inoculation method was based on this route, and resulted in MRSA colonization in contact pigs. However, in our experiment we cannot distinguish between direct faecal-oral contact and indirect contact via the environment. After placing MRSA positive C_1-pigs together with MRSA negative C_2-pigs in a 'clean' environment, the number of MRSA positive pigs first declined from four pigs at day 21 to one pig at day 30. After that, the number of MRSA positive pigs increased to all nine pigs from day 41 till day 48. MRSA concentration, i.e. infection pressure, seems to decrease initially, after which the infection pressure increases and then stabilizes. This might indicate that environmental contamination does play a role in MRSA transmission.

Absence of antimicrobial use in both our experiments indicates that antimicrobials are not required for MRSA colonization and –transmission to occur in pigs. This was also demonstrated in a longitudinal survey on a pig farm in Canada, where MRSA was present in piglets and sows for longer periods in the absence of antimicrobial use (Weese et al., 2011). Reduction of antimicrobial use seems to be a necessary but insufficient condition to eradicate MRSA on pig farms. Other risk factors and interventions need to be evaluated to come to an effective control program. Our method to quantify MRSA transmission can be used to evaluate the effect of interventions on MRSA transmission by comparing the levels of transmission under different experimental conditions (De Jong and Kimman, 1994).

The current estimate of R_0 was based on a single *spa* type, i.e. t011. Although, *spa* type t011 is by far the predominant spa type of LA-MRSA in the European Union (EFSA, 2009), possible strain-dependent infectivity might influence the transmission capability.

To conclude, the results of both experiments showed that the mechanisms behind MRSA colonization are still to be elucidated. The transmission experiment proved that LA-MRSA is able to spread easily among pigs and to persist in pig populations, even without antimicrobial use.

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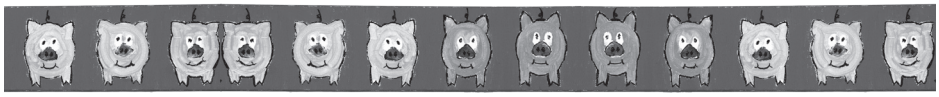
CHAPTER 4.2

LONGITUDINAL FIELD STUDY ON WITHIN-HERD TRANSMISSION OF LIVESTOCK-ASSOCIATED METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN PIGS



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submitted in slightly adapted version

KEYWORDS

- Methicillin resistant
- *Staphylococcus aureus*
- Pigs
- Transmission
- Longitudinal
- Reproduction ratio
- MRSA

ABSTRACT

Since the detection of MRSA CC398 in pigs in 2004, it has emerged in livestock worldwide. This study assessed MRSA longitudinally in 2 Danish and 4 Dutch pig herds, and quantified MRSA transmission within pig herds and factors affecting it. Sows and their pigs were sampled at varying intervals during a production cycle. Prevalences from sows increased from 33% before farrowing to 77% just before weaning. Prevalences from pigs were > 60% during the entire study. The recurrent finding of MRSA in the majority of sampled individuals either indicates prolonged or persistent colonization or might be the result of repeated contamination. Transmission rates and with that values of the reproduction ratio *(R_0)*, based on multivariable analysis, varied from 0.24 to 8.08. Transmission rates were higher when tetracyclins and β-lactams were used, indicating a selective advantage of MRSA CC398 when these antimicrobials are used. Furthermore, transmission rates were higher in pre-weaning pigs than in post-weaning pigs, indicating an age-related susceptibility or an effect of the sow. Transmission rates increased with the relative increase of the infection pressure within the pen compared to the total infection pressure, implying that transmission through direct contact with pen mates is a more important transmission route compared to transmission through indirect contact with other animals within the section or with its environment. The results indicate that MRSA CC398 is able to spread and persist in pig herds, resulting in an endemic situation.

INTRODUCTION

In 2004, a distinct clone of methicillin resistant *Staphylococcus aureus* (MRSA CC398), referred to as livestock-associated (LA), was found in pigs and in people in contact with pigs (Voss et al., 2005). Various observational studies have detected LA-MRSA in pig and other livestock herds worldwide, and risk factors for herds to be MRSA positive have been identified (EFSA, 2009; Smith et al., 2009; Graveland et al., 2010; Mulders et al., 2010; Broens et al., 2011b, 2011c).

Antimicrobial resistant micro-organisms in livestock become a public health issue when resistant organisms transfer from livestock to humans. The role of animal populations in the transmission of micro-organisms to humans is not only dependent on the possibility of transmission from animals to humans, but also on the possibility of transmission between animals.

The primary route of MRSA transmission between humans seems to be direct contact with individuals carrying MRSA (Eveillard et al., 2004; Albrich and Harbarth, 2008). However, environmental spread might be a substantially underestimated route for MRSA transmission in hospitals (Sexton et al., 2006; Boyce, 2007). Similar mechanisms seem likely for MRSA transmission between pigs. MRSA is not only isolated from nasal samples taken from individual pigs, but also from environmental samples taken on pig herds (EFSA, 2009; Broens et al., 2011a, 2011b), indicating possibilities for direct and indirect transmission. Little is known about MRSA–transmission and factors affecting it within pig herds and about duration of MRSA colonization over time. A limited study in one pig herd assessed MRSA colonization in piglets over time and showed age-related differences in MRSA prevalence in young pigs (< 10 weeks) (Weese et al., 2011).

Transmission can be measured in longitudinal field studies and experiments, and can be expressed with the reproduction ratio *(R_0)*, which is an essential parameter in management of diseases. R_0 is defined as the average number of secondary cases caused by one typical infectious individual during its entire infectious period in a completely susceptible population, and is often used as a quantitative measure of transmission (Kermack and McKendrick, 1991; De Jong and Kimman, 1994). R_0 has a threshold value of 1; if $R_0 > 1$, minor and major outbreaks can occur and an endemic situation can be established and maintained, whereas when $R_0 < 1$ an infection does not spread and will not become endemic, i.e. the infection will fade out (De Jong and Kimman, 1994; Velthuis et al., 2007).

The objective of this study was to determine MRSA over time in Danish and Dutch pig herds and to quantify transmission rates and routes and factors affecting it for MRSA between pigs.

MATERIAL AND METHODS

Selection of herds and sampling

Six farrow-to-finish herds, confirmed MRSA positive, were selected by convenience: 2 Danish (DK1, DK2; selected from a Danish pilot study, and 4 Dutch herds (NL1, NL2, NL3, NL4; selected from a cross-sectional prevalence study (Broens et

al., 2011b). Per herd, 1 cohort of sows due to farrow in a selected time period and in the same farrowing section, was selected for sampling, except for herd NL4, where two cohorts of sows were included (NL4a and NL4b) with a time interval of 3 months.

Sows and their offspring were sampled several times during a production cycle. Nasal swabs were taken from all pigs at all sampling moments. Additionally, vaginal swabs were taken from the sows at all sampling moments, and rectal swabs were taken from new born pigs (moment 2; Table 1). At all sampling moments, 4-5 environmental wipes (Sodibox, s1 kit ringer solution, France) were taken from surfaces in the selected herd sections.

In total, 63 sows and their pigs were included at the start of the study. The number of pigs at each sampling moment varied depending on the number of pigs born alive and on movement or death of pigs. After weaning, sows returned to the breeding section and were omitted from further sampling. The cohorts of pigs were monitored until just before slaughter. Efforts were made to track down all pigs during the entire study period, but in some herds, (groups of) pigs were lost for follow up due to sorting and mixing of pigs. In one Dutch herd (NL1), samples from sampling moment 5 and 6 were missing, because the pigs were moved to another location.

Table 1. Sampling moment, section, approximate time in life, age group and type of samples taken per herd for MRSA isolation.

Moment[a]	Section	Approximate time in life	Age group	Type of sample	
				Sows	Pigs
1	Farrowing	1 wk before birth	Sows	Nasal/vaginal	-
2	Farrowing	3 days after birth	Sows/pigs	Nasal/vaginal	Nasal/rectal
3	Farrowing	3 wks after birth	Sows/pigs	Nasal/vaginal	Nasal
4	Weaning	6 wks after birth	Pigs	-	Nasal
5	Weaning	10 wks after birth	Pigs	-	Nasal
6	Finishing	25 wks after birth	Pigs	-	Nasal

[a] Each sampling moment, 4-5 environmental wipes were taken in each section

Microbiological analysis

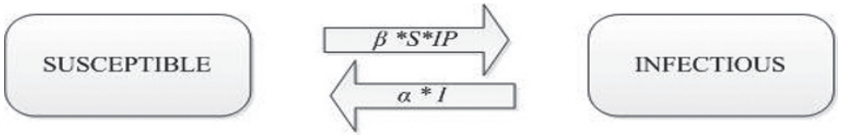
Samples were enriched using Mueller Hinton Broth with 6.5% NaCl (MHB+). Nasal, vaginal and rectal swabs were placed into 10 mL MHB+, environmental wipes into 100 mL MHB+. After 18h of aerobic incubation at 37°C, a loop-full of MHB+ was spread onto sheep blood agar (Oxoid, PB5008A, UK) and a chromogenic MRSA screen agar (Oxoid, PO5196A, UK). One suspected colony per sample

was confirmed to be MRSA CC398 by two PCR's (De Neeling et al., 1998; Stegger et al., 2011).

Data analysis

To estimate transmission parameters it is essential to determine the MRSA status of individual pigs at each sampling moment. For each moment, pigs were classified infectious *(I)* if either one of the swabs (nasal, rectal or vaginal) tested positive for MRSA. Pigs were classified susceptible *(S)* if all swabs tested negative for MRSA. New born pigs were assumed to be MRSA negative, thus susceptible, at birth.

Based on data from a former experimental study (Broens et al., accepted) and on the fact that most humans are intermittent carriers (Van Belkum et al., 2009), it was assumed that infectious pigs stop shedding after a while and become susceptible again. Therefore, a susceptible-infectious-susceptible *(SIS)* model (Velthuis et al., 2007) was used to describe the transmission of LA-MRSA within herds. MRSA is spread via the environment or by direct contact between infected and susceptible individuals; these individuals can be pen mates (within-pen transmission) or individuals in other pens within the section (between-pen transmission). Three sources of MRSA for a susceptible pig were allocated in our study: infectious individuals, including sows, within the pen, infectious individuals, including sows, within the section (but not in the same pen), and the environment. The term total infection pressure (IP) was introduced and defined as the sum of the proportion of infectious pigs within the pen (IP within the pen), the proportion of infectious pigs within the section, but not in the same pen (IP other pens), and the proportion of positive environmental wipes (IP environment). The *SIS*-model can then be represented as follows:



In this model, β is the transmission parameter, defined as the number of secondary cases *(C)* out of a number of susceptible individuals *(S)* caused by a certain infection pressure (IP) during each time interval between samplings *(Δt)*. The number of new cases *(C)* per time interval *(Δt)* depends directly on β, *S* and IP; infectious individuals *(I)* become susceptible again at recovery rate α. *C, I, S* and *Δt* were determined per pen. Underlying assumptions were that (1) all pigs within a pen were randomly in contact, (2) susceptible and infectious individuals were homogenous groups, i.e. all individuals were equally susceptible or infectious, and (3) infectious individuals were equally infectious over time. Data were statistically analysed with SAS version 9.1 software using Generalized Linear Models (PROC GENMOD) (SAS, 2004) with a complementary log-log link function, the term log (IP*Δt) as offset, *C* as the number of new cases, and *S* as the number of trials in the binomial process. The relation between the expected value (E) and the offset

and explanatory variable is presented in the following statistical model, which is an 'intercept only' model:

$$c \log \log (E\ C/S) = \log (\beta) + \log (IP^{*}\Delta t)$$

Exponentiation of the estimated parameter log (β) gives the transmission parameter β, that denotes the transmission per day. The reproduction ratio R_0 can then be calculated by multiplying β with the length of the infectious period ($1/\alpha$). We assumed 17.4 days to be the length of the infectious period. This was based on the average length of the infectious period observed in a transmission experiment (Broens et al., accepted) and on the interval of sampling in this study, which ranged from 4 to 94 days.

Information on antimicrobial use per animal was registered during the study. For the statistical model, explanatory variables potentially affecting transmission were added to the intercept only model. These variables were related to antimicrobial use, age and contribution of direct and indirect transmission. Tetracyclins and β-lactam antimicrobials were defined as risk antimicrobials (ab), as these antimicrobial classes select 100% for MRSA CC398 (Kadlec et al., 2009) and will therefore select for MRSA and potentially affect its transmission. If these antimicrobials were applied on > 1 pig within a pen during a time interval, then variable 'ab' was defined as yes, otherwise no. Age of pigs was introduced as variable, because an age-effect or presence of the sow in the farrowing section might affect transmission of MRSA compared to transmission in post-weaning pigs. When pigs were located in the farrowing section, 'age' was defined as pre-weaning, and after weaning when located in the weaning or finishing section as post-weaning. To quantify the relative effect of transmission through direct contact with pen mates compared to the total transmission through direct and indirect contact, a continuous explanatory variable was introduced. This variable (pIP) was calculated as IP within the pen divided by the total IP.

Data from Denmark and The Netherlands were analysed separately and in combination. First, analysis was done without explanatory variables to estimate a basic transmission parameter (Table 2). Secondly, analysis including explanatory variables was done. Due to very high prevalences in new born pigs in both Danish herds, the number of susceptibles was very low after sampling moment 2, leaving no or very few cases to occur at post-weaning age. Analysis was, therefore, done on data from pre-weaning pigs only, implying that the explanatory variable 'age' could not be included in this analysis (Table 2). Finally, multivariable analysis was performed on Dutch data only from all pigs.

In bi- and multivariable analysis two-way interactions between variables were tested for significance and removed if $P > 0.05$. To estimate solely the herd effect, i.e. without explanatory variables, herd was included in the 'intercept only' model as random effect using an exchangeable covariance structure. Herd effect for Dutch data only accounted for 0.06% of non-explained variance, and was therefore not included in the statistical models. Herd effect for Danish data could not be estimated.

Table 2. Formulas for transmission parameter estimation and used data for three models used to quantify transmission of MRSA within pig herds.

Model	Transmission parameter estimation[a]	Used data
Intercept only	$TP_1 = \exp(\log \beta_0)$	Denmark / Netherlands
Bivariable	$TP_2 = \exp(\log \beta_0 + \log \beta_1(\text{ab}) + \log \beta_2(\text{pIP}))$	Denmark / Netherlands
Multivariable	$TP_3 = \exp(\log \beta_0 + \log \beta_1(\text{ab}) + \log \beta_2(\text{pIP}) + \log \beta_3(\text{age}))$	Netherlands

ab = use of tetracyclins or β-lactams (yes/no); age = age of pigs (pre-weaning / post-weaning);
pIP = infection pressure within the pen divided by total infection pressure (continuous variable)
[a] In all models log (IP*Δt) was used as offset variable

RESULTS

MRSA prevalence

Overall MRSA prevalence of sows increased from 33.3% (exact 95% CI: 22.0-46.3) before farrowing (moment 1), to 58.8% (exact 95% CI: 46.2-70.6) after farrowing (moment 2) and to 77.3% (exact 95% CI: 65.3-86.7) ~3 weeks later, just before weaning (moment 3). All sows in herd NL4 tested MRSA negative, whereas all sows in herd DK1 tested MRSA positive at all sampling moments. Around farrowing (moment 1 and 2), MRSA prevalences in Danish sows were significantly higher ($P < 0.0001$) than in Dutch sows. At weaning (moment 3), MRSA prevalences of Danish and Dutch sows were not different (Table 3).

Pre-weaning, overall MRSA prevalence of pigs increased from 60.9% (exact 95% CI: 57.4-64.3) in new born pigs (moment 2) to 77.8% (exact 95% CI: 74.6-80.7) ~3 weeks later, just before weaning (moment 3). Post-weaning, MRSA prevalence of pigs was 79.6% (exact 95% CI: 76.2-82.8) at moment 4 and 86.6% (exact 95% CI: 83.2-89.5) at moment 5, both in the weaning section. In the finishing section, just before slaughter, MRSA prevalence was 69.6% (exact 95% CI: 64.9-74.1). At all moments, MRSA prevalences in Danish pigs were significantly higher ($P < 0.0001$) than in Dutch pigs (Table 4).

New born pigs (moment 2) in Dutch herds were more often MRSA positive ($P < 0.0001$) if their dam was MRSA positive before farrowing compared to MRSA negative dams. MRSA prevalence in pigs from positive dams was 84% versus 48% from negative dams. In Danish herds, this association was not present ($P = 0.61$). MRSA prevalence in pigs from positive dams was 78% versus 73% from negative dams. Combining data from all herds, 81% of new born pigs from MRSA positive sows were positive after birth, whereas 50% of pigs from MRSA negative sows were positive at that time ($P < 0.0001$).

The number of MRSA positive environmental wipes varied largely between herds and sampling moments, from no positive wipes to all wipes positive (Table 3 and 4).

Table 3. Total number and number of positive environmental wipes, number of sows and percentage positive[a] for sampling moments 1-3 per herd, all Dutch (NL) and Danish (DK) herds and for all herds (NL+DK).

	Moment 1			Moment 2			Moment 3		
	Wipes	Sows		Wipes	Sows		Wipes	Sows	
Herd	*n* pos / *n* total	*n*	% pos	*n* pos / *n* total	*n*	% pos	*n* pos / *n* total	*n*	% pos
NL1	1/4	16	6.3	3/4	16	25.0	4/4	16	93.8
NL2	4/4	12	33.3	4/4	12	100.0	4/4	12	100.0
NL3	3/4	8	50.0	4/4	8	62.5	4/4	8	100.0
NL4a	1/4	6	0.0	0/4	6	0.0	0/4	4	0.0
NL4b	0/4	6	0.0	0/4	6	0.0	0/4	6	0.0
NL	9/20	48	18.8	11/20	48	43.8	12/20	46	76.1
DK1	5/5	5	100.0	5/5	6	100.0	10/10	6	100.0
DK2	5/5	10	70.0	4/5	14	92.9	5/5	14	71.4
DK	10/10	15	80.0	9/10	20	95.0	15/15	20	80.0
NL+DK	19/30	63	33.3	20/30	68	58.8	27/35	66	77.3

[a] Sows were classified MRSA positive if either one of the swabs (nasal/vaginal) tested positive; from all MRSA positive samplings of sows (n=112), 58% was classified positive based on both swabs, 39% on a positive nasal swab and 3% on a positive vaginal swab.

Table 4. Number of pigs and percentage positive[a] for sampling moments 2-6[a] and total number and number of positive environmental wipes for sampling moments 4-6 per herd, all Dutch (NL) and Danish (DK) herds and for all herds (NL+DK).

	Moment 2		Moment 3		Moment 4			Moment 5			Moment 6		
	Pigs		Pigs		Wipes	Pigs		Wipes	Pigs		Wipes	Pigs	
Herd	*n*	% pos	*n*	% pos	*n* pos /*n* total	*n*	% pos	*n* pos /*n* total	*n*	% pos	*n* pos /*n* total	*n*	% pos
NL1	199	42.7	186	93.0	4/4	125	100.0	-	-	-	-	-	-
NL2	147	100.0	146	99.3	0/4	93	55.9	2/4	92	100.0	4/4	80	86.3
NL3	90	95.6	90	98.9	4/4	87	100.0	4/4	86	100.0	4/4	82	63.4
NL4a	68	4.4	64	3.1	0/4	65	52.3	1/4	64	89.1	3/4	62	64.5
NL4b	79	1.3	70	0.0	1/4	67	20.9	0/4	68	16.2	0/4	68	13.2
NL	583	55.2	556	73.6	9/20	437	71.4	7/16	310	79.4	11/16	292	58.2
DK1	57	100.0	48	100.0	10/10	48	100.0	7/10	45	100.0	5/5	46	100.0
DK2	142	68.3	133	87.2	10/10	129	100.0	15/15	123	100.0	10/10	67	98.5
DK	199	77.4	181	90.6	20/20	177	100.0	22/25	168	100.0	15/15	113	99.1
NL+DK	782	60.9	737	77.8	29/40	614	79.6	29/41	478	86.6	26/31	405	69.6

[a] Pigs were classified MRSA positive if either one of the swabs (nasal/rectal) tested positive; from all MRSA positive samplings of pigs (n=476), 67% was classified positive based on both swabs, 31% on a positive nasal swab and 2% on a positive rectal swab.

Transmission quantification

Table 5 shows model input summarized per herd per sampling interval, which was used for MRSA transmission quantification.

Basic R_0-values including data from all pigs in the 'intercept only' model, were 1.11 (95% CI: 1.00-1.22) for Dutch pigs and 1.88 (95% CI: 1.59-2.22) for Danish pigs. Including data from pre-weaning pigs only, resulted in a slightly higher R_0-value for Dutch pigs (R_0 = 1.96; 95% CI: 1.74-2.20), whereas the R_0-value for Danish pigs did not change (R_0 = 1.88; 95% CI: 1.59-2.22). Analysis on data from both countries resulted in intermediate R_0-values (Table 6).

Bivariable analysis of data from pre-weaning pigs only, showed effects of both variables: use of risk antimicrobials (ab: $P < 0.0001$ for Dutch data and $P = 0.10$ for Danish data) and the relative proportion of IP within the pen compared to the total IP (pIP: $P < 0.0001$ for Danish and Dutch data), and no significant interaction effect ($P > 0.05$). The effect of pIP was very large, especially in Danish herds. For Danish pigs, R_0 was 0.02 (95% CI: 0.00-0.07) without use of risk antimicrobials and pIP at its minimum (i.e. 0), and 0.03 (95% CI: 0.00-0.16) with use of risk antimicrobials and pIP at its minimum. When pIP was at its maximum, R_0-values

were infinitely large both without and with use of risk antimicrobials (R_0 = 121076 (95% CI: 307.31-4.77E+07) and 1760410 (95%CI: 287.92-1.08E+08), respectively). For Dutch pigs, R_0–values were 0.62 (95% CI: 0.45-0.84) without use of risk antimicrobials and pIP at its minimum, and 1.54 (95% CI: 0.84-2.83) with use of risk antimicrobials and pIP at its minimum. When pIP was at its maximum, R_0-values were 3.28 (95% CI: 1.43-7.49), and 8.17 (95% CI: 2.65-25.22) for without and with use of risk antimicrobials, respectively (Table 7). Analysis on data from both countries resulted in similar results, with the lowest R_0 when risk antimicrobials were not used and pIP at its minimum (R_0 = 0.68; 95% CI: 0.54-0.85), and the highest R_0 when risk antimicrobials were used and pIP at its maximum (R_0 = 10.50; 95% CI: 4.31-25.59). Average pIP was 0.32 (SD = 0.21) for Dutch herds and 0.27 (SD = 0.08) for Danish herds (Table 5).

Multivariable analysis of all Dutch data showed significant effects of all three variables, ab, age and pIP ($P < 0.0001$); interaction effects were not significant ($P > 0.05$). Figure 1 shows the effect of ab and age on R_0 for pIP-values between 0 and 1. R_0 was lowest at 0.24 (95% CI: 0.18-0.31) when no risk antimicrobials were used in post-weaning pigs and pIP = 0. R_0 increased to 0.60 (95% CI: 0.34-1.06) when risk antimicrobials were used in post-weaning pigs and a minimal pIP. R_0 was above 1, though not significant (R_0 = 1.56; 95% CI: 0.65-3.77), when risk antimicrobials were used in pre-weaning pigs and a minimal pIP, and below 1, though not significant, (R_0 = 0.61; 95% CI: 0.34-1.10) when risk antimicrobials were used in post-weaning pigs and a minimal pIP. R_0 increases with increasing pIP. Given a maximal pIP of 1, R_0 was 1.22 (95% CI: 0.60-2.48) without using risk antimicrobials in post-weaning pigs and significantly above 1, i. 3.12 (95% CI: 1.15-8.46) when risk antimicrobials were used in this age-group. In pre-weaning pigs, R_0-values were significantly above 1; R_0 was 3.16 (95% CI: 1.14-8.82) when no risk antimicrobials were used, and highest with use of risk antimicrobials (R_0 = 8.08; 95% CI: 2.17-30.12).

Table 5. Summarized model input per herd used for estimation of MRSA transmission parameters for Danish (DK) and Dutch (NL) herds.

[a] Interval I –V = time between subsequent sampling moments starting from birth

[b] Number of pens used in statistical analysis

[c] Number of pens where > 1 pig received treatment with risk antimicrobials, i.e. tetracyclins or β-lactams

[d] NL: mean = 0.32; SD = 0.21, DK: mean = 0.27; SD = 0.08

– = no susceptibles left

Table 5.

Herd	Interval[a]	Infectious (*n*/section)	Susceptible (*n*/section)	Cases (*n*/section)	Δt (days)	Pens[b] (*n*)	Risk ab[c] (*n* pens)	pIP (range)
NL1	I	0	199	85	4	16	9	0.00-0.47
	II	34	56	50	14	10	4	0.28-0.34
	III	92	11	11	21	5	5	0.33-0.33
	IV	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-
NL2	I	0	147	147	6	12	12	0.33-0.33
	II	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-
	IV	36	40	40	21	10	0	0.40-0.40
	V	-	-	-	-	-	-	-
NL3	I	0	90	86	5	8	8	0.28-0.34
	II	17	4	4	14	2	0	0.33-0.33
	III	42	1	1	21	1	0	0.33-0.33
	IV	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-
NL4a	I	0	68	3	2	6	0	0.00-0.91
	II	3	61	1	21	6	1	0.00-1.00
	III	2	62	31	13	6	0	0.23-0.70
	IV	23	30	23	21	5	0	0.33-0.47
	V	16	2	1	80	2	0	0.20-0.34
NL4b	I	0	79	1	4	6	0	0.00-1.00
	II	-	-	-	-	-	-	-
	III	0	67	14	24	6	0	0.00-0.51
	IV	14	53	7	15	6	0	0.30-0.66
	V	4	19	8	94	2	0	0.12-0.88
NL		283	989	513		109	39	0.00-1.00[d]
DK1	I	0	57	57	3	5	0	0.31-0.31
	II	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-
	IV	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-
DK2	I	0	142	97	4	14	14	0.05-0.37
	II	27	41	34	18	7	0	0.23-0.32
	III	86	17	17	22	7	1	0.22-0.22
	IV	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-
DK		113	257	205		33	15	0.05-0.37[d]

Table 6. Transmission per day from the 'intercept only model' (TP1) and R_0-valuea with its 95% confidence interval for Dutch (NL) and Danish (DK) herds separately and all herds (NL+DK)for pre-weaning pigs only and for all pigs.

	Pre-weaning pigs only				All pigs			
Country	*C/S*[b]	TP_1	R_0	95% CI	*C/S*[b]	TP_1	R_0	95% CI
NL	377/704	0.112	1.96	1.74 - 2.20	513/989	0.064	1.11	1.00 - 1.22
DK	188/240	0.108	1.88	1.59 - 2.22	205/257	0.108	1.88	1.59 - 2.22
NL+DK	565/944	0.111	1.93	1.75 - 2.13	718/1246	0.071	1.24	1.14 - 1.35

[a] To calculate R_0, TP_1 was multiplied with 17.4 days (= length of infectious period)
[b] Number of MRSA cases *(C)* from total number of susceptibles *(S)* used in analysis

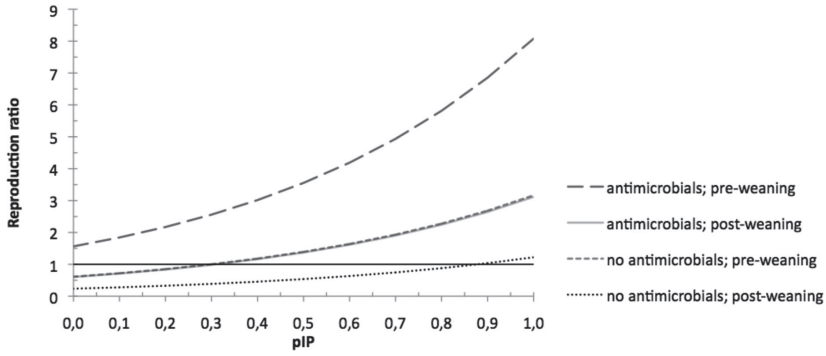


Figure 1. The reproduction ratio for MRSA CC398 in Dutch pigs, for use of tetracyclins and β-lactam antimicrobials (yes, no), age of pigs (pre-weaning, post-weaning) related to the relative proportion of the infection pressure within the pen compared to the total infection pressure (pIP); based on 513 MRSA cases *(C)* from 989 susceptibles *(S)* from 4 herds.
To calculate the reproduction ratio, estimated transmission parameters were multiplied with 17.4 days (= length of infectious period)
Note: lines for antimicrobials; post-weaning pigs and no antimicrobials; pre-weaning pigs are overlapping, thus difficult to distinguish.

Table 7. Transmission per day resulting from bivariable analysis with use of risk antimcrobials[a] and the relative proportion of IP compared to the total IP (pIP) included in the model[b] *(TP2)* and R_0-value[c] with its 95% confidence interval for Dutch (NL) and Danish (DK) farms separately and all farms combined (NL+DK) for pre-weaning pigs only.

		No risk antimicrobials; pIP=0			Risk antimicrobials; pIP=0			No risk antimicrobials; pIP=1			Risk antimicrobials; pIP=1		
Country	*C/S*[d]	TP_2	R_0	95% CI	TP_2	R_0	95% CI	TP_2	R_0	95% CI	TP_2	R_0	95% CI
NL	377/704	0.035	0.62	0.45 - 0.84	0.089	1.54	0.84 - 2.83	0.188	3.28	1.43 - 7.49	0.470	8.17	2.65 - 25.22
DK	188/240	0.001	0.02	0.00 - 0.07	0.002	0.03	0.00 – 0.16	6958	121076	307.31 - ∞	10117	176041	287.92 - ∞
NL+DK	565/944	0.039	0.68	0.54 - 0.85	0.076	1.32	0.84 - 2.06	0.309	5.38	2.74 – 10.56	0.603	10.50	4.31 - 25.59

[a] Risk antimicrobials (tetracyclins and β-lactams) in > 1 pig per pen

[b] Interaction between variables was not significant ($P > 0.05$)

[c] To calculate R_0, TP_2 was multiplied with 17.4 days (= length of infectious period)

[d] Number of MRSA cases *(C)* from total number of susceptibles *(S)* used in analysis

DISCUSSION

Prevalences and transmission rates of MRSA between pigs were assessed longitudinally in 6 herds in 2 European countries. Prevalences varied widely between herds and over time. Prevalence in sows before farrowing was much higher in Denmark (80%) than in The Netherlands (19%). This might be explained by differences in management practices. In The Netherlands, an all in – all out system is applied in the farrowing section, i.e. a cohort of sows due to farrow is placed into a 'clean' farrowing section, whereas in Denmark a more continuous system is practiced. Except for 1 Dutch herd, prevalence in sows increased in The Netherlands during time in the farrowing section, which might be explained by a build-up of bacterial load, i.e. the infection pressure, during time spent in this section.

Prevalences in pigs just after birth varied from 1 to 100%, Dutch pigs showing the lowest overall prevalence (55% versus 78% in Denmark). A similar explanation as given for differences in prevalences in sows might be applicable here. In 1 Dutch herd, prevalences in pre-weaning pigs remained low (< 5%). A rapid increase in prevalence in this herd was seen after weaning, despite the fact that no risk antimicrobials were used during that time interval and that no positive dust samples were found at the first sampling moment in the weaning section. In a longitudinal study on an antimicrobial-free Canadian pig farm, an increase in MRSA prevalence around the time of weaning was seen as well (Weese et al., 2011). Co-mingling of MRSA positive and negative pigs, transmission through human handling during weaning, an increased susceptibility due to stress or related to age, or a combination of these factors, might have caused the rapid increase in MRSA prevalence after weaning.

The recurrent finding of MRSA in the majority of sampled individuals either indicates prolonged or persistent colonization, or might be the result of repeated contamination. To distinguish between persistent colonization and repeated contamination, MRSA positive pigs should be placed in a clean environment individually for a longer time.

The basic reproduction ratio, based on a *SIS*-model without explanatory variables, was significantly above one, indicating a high probability of transmission and persistence within a pig herd (Allen and Burgin, 2000). The reproduction ratios were calculated by multiplying estimated transmission parameters with the length of the infectious period as measured in an earlier experiment (Broens et al., accepted). An infectious period is only valid for infectious individuals. As MRSA can survive outside the host for long periods (Quinn et al., 2002), the environment might also be a source of MRSA. How the contamination of the environment reflects the prevalence of pigs in this environment, how long LA-MRSA persists in the environment, and how environmental contamination affects transmission, is unknown. Our method for quantification of the reproduction ratio might, therefore, be less applicable in situations where no or very few pigs are MRSA positive in a section, and where MRSA is only present in the environment. For our calculations we used 17.4 days as the length of the infectious period. The final observatio-

nal time interval, i.e. the period in the finishing pig section, in particular, was much longer (> 10 weeks). During this period, more than one infection might have occurred in one individual, whereas our method only counts one. This implies a potential underestimation of transmission rates. Based on the prevalence in pigs just before slaughter (70%), the reproduction ratio can be estimated using R_0 = 100/(100-prevalence) (Allen and Burgin, 2000), resulting in 3.27. This is indeed higher than the estimated basic reproduction ratio based on data from all pigs in both countries (R_0 = 1.24), indicating an underestimation, however, it is similar to the estimated reproduction ratio from our transmission experiment (Broens et al., accepted).

Transmission rates were higher when tetracyclins and β-lactams were used which might be explained by a selective advantage of MRSA CC398 as compared to susceptible strains when these antimicrobials are used. An experimental study investigating the effects of zinc and tetracycline on MRSA counts in nasal samples of pigs, showed higher counts in treated animals than in untreated animals, which seems to confirm a selective advantage of MRSA CC398 caused by both compounds (Moodley et al., 2011b). The effect of zinc could not be assessed in our study as only one herd, a Danish herd, applied zinc in the weaning section where all pigs already were infectious with no susceptibles left.

The proportion of susceptibles and therefore, potentially new cases, was relatively high among pre-weaning pigs compared to post-weaning pigs; we actually assumed all new born pigs to be MRSA negative, and thus susceptible. MRSA prevalence in post-weaning pigs was much higher, leaving fewer susceptibles to become a case and thus estimation of transmission rates was based on less information in this age-group. Thus the power of the comparison is lower. Nevertheless, the available data indicated that transmission rates in pre-weaning pigs are significantly higher than in post-weaning pigs. This might be explained by the presence of the sow, which might be a primary source of MRSA for the new born pigs. An association between MRSA status of the sow prior to farrowing and the MRSA status of a pig after birth was shown in our study, which was found in a longitudinal study on a Canadian pig farm as well (Weese et al., 2011). Transmission of MRSA CC398 from sow to pigs was also shown in an experimental setting (Moodley et al., 2011a). Additionally, young pigs might be more susceptible to infections due to an immature mucosal immune system, or a greater impact of antimicrobials on their unbalanced microbiota (Zoetendal et al., 2004a; Bailey et al., 2005).

The increase of transmission rates with the increase of the infection pressure within the pen relative to the total infection pressure implies that transmission through direct contact with pen mates is an important transmission route. More quantitative information on transmission rates for within- and between-pen transmission can be obtained by transmission experiments (Klinkenberg et al., 2002). To assess the role of environmental contamination in transmission and persistence of MRSA in pig populations more information is needed.

Since only 4-6 farrow-to-finish herds were included in the estimation of transmission rates, including some herds with very high prevalences leaving just a few trials for

parameter estimation, the results might not be representative for the international pig herd population. Moreover, the observed association between explanatory variables and R_0, e.g. antimicrobial use, might be confounded by other effects. Although herd effect accounted for only 0.06% of the variance in the multivariable analysis, it was not possible to distinguish the herd effect from an unconfounded estimate of the exposure effects. Prudence is therefore called for in drawing conclusions on these associations and studies involving larger numbers of herds are required to confirm our findings.

To summarize, introduction of MRSA in a fully susceptible population most probably leads to transmission and an endemic situation with direct contact between animals as most important route of transmission. Control programs should therefore focus on (1) prevention of introduction into a herd, and (2) prevention of transmission within a herd, e.g. by restrictive antimicrobial use and hygiene barriers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MJ, EG, AG, LG, PB and EB contributed to the design of the longitudinal field study. EB, PW and CEG coordinated and performed the studies. EG, NV, MJ and EB analysed the data. EB, EG and CEG drafted the manuscript. All authors read and approved the final manuscript.

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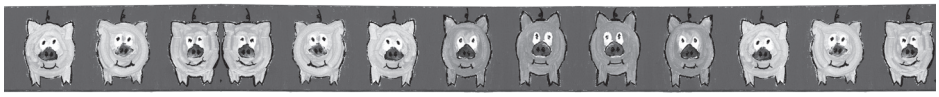
GENERAL DISCUSSION



INTRODUCTION

Antimicrobial resistance in bacteria from pigs becomes a public health issue when resistant bacteria or their resistance genes transfer from pigs to humans. The use of antimicrobials in pigs can result in selection and transmission of resistant clones, which might lead to outbreaks and epidemics, and possibly to an endemic situation within the pig population (Fig. 1). Depending on the host specificity of the bacterium, outbreaks might be confined to a single animal species. In the case of potential zoonotic bacteria, outbreaks in pigs can lead to human infections with potential treatment failure. Additionally, resistant bacteria can transfer their resistance genes to other bacteria belonging to human commensal flora (Acar and Rostel, 2001; Aubry-Damon et al., 2004).

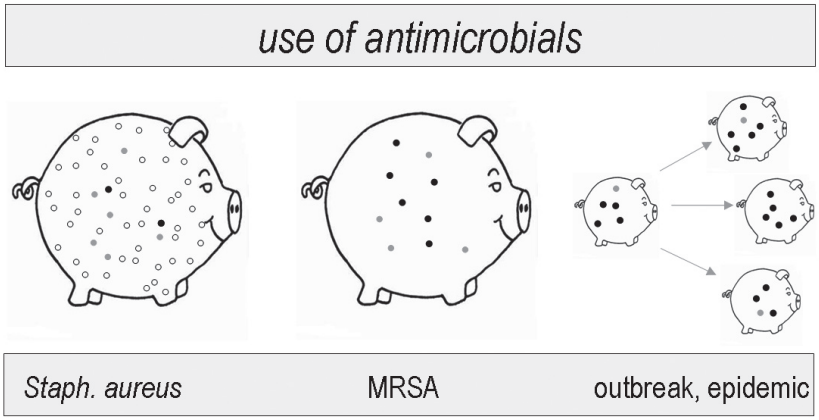


Figure 1. Model of selection for and transmission of MRSA in pigs and the impact of exposure to antimicrobials (adapted from SWAB, 2003)

Methicillin resistant *Staphylococcus aureus* (MRSA) is the most important cause of antimicrobial resistant health-care associated infections in humans worldwide (EARSS, 2007). Traditionally, MRSA was confined to hospitalized patients, but the last decades MRSA infections have occurred more frequently in the general public (Chambers, 2001; Kluytmans-Vandenbergh and Kluytmans, 2006), and in 2004 an association with livestock was detected. MRSA was isolated from pigs, veal and poultry and transmission to humans in contact with these animals was confirmed (Voss et al., 2005; Van Den Broek et al., 2009; Graveland et al., 2010; Mulders et al., 2010). It was concluded that livestock is a zoonotic reservoir for MRSA CC398, now-called livestock-associated (LA-)MRSA.

Antimicrobial use in pigs will select for LA-MRSA, and through direct and indirect contacts transmission within and between herds will occur. Transfer to humans, subsequently, is possible through direct contact with positive pigs, environmental or airborne transmission, or handling and consuming contaminated food.

In The Netherlands, antimicrobial use in human medicine is among the lowest in Europe (Fig. 2; Coenen et al., 2009), whereas antimicrobial use in veterinary

medicine is among the highest (Fig. 3; Grave et al., 2010). The use of antimicrobials among food-producing animals, expressed in mg active substances per total kg biomass of meat, is highest for pig meat.

The key to limiting LA-MRSA transmission from pigs to humans is to reduce the transmission of LA-MRSA within and between pig populations. The research described in this thesis aimed to gain more insight into the occurrence and transmission dynamics of LA-MRSA in pig populations in order to identify (cost-) effective strategies for infection control in pigs, and with that to prevent the transfer to humans. Results and potential interventions will be discussed in this chapter focusing on transmission between and within pig herds, transmission between pigs and humans and finally transmission between humans. Recommendations for the future and a summary of the main conclusions are presented at the end.

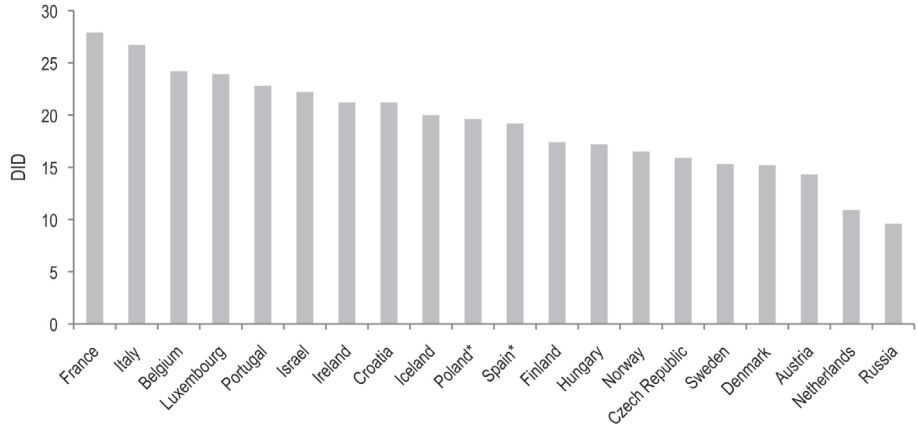


Figure 2. Outpatient antibiotic use in 20 European countries in 2006 in defined daily doses per 1000 inhabitants per day (DID). * 2005 data (adapted from Coenen et al., 2009)

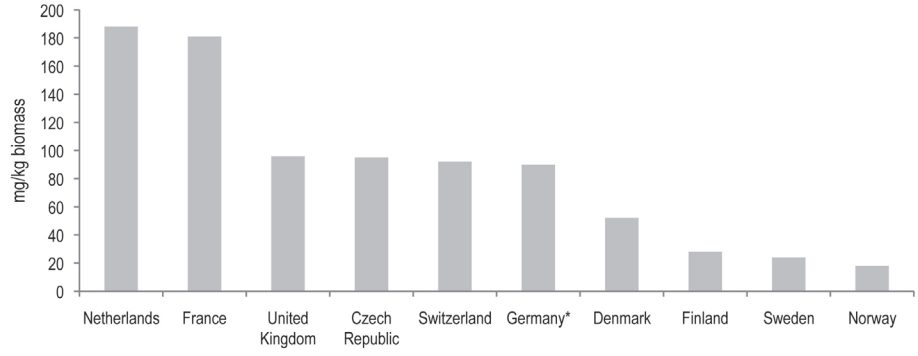


Figure 3. Amounts, in mg, of veterinary antibacterial agents sold in 2007 per kg biomass of pig meat, poultry meat and cattle meat produced plus estimated live weight of dairy cattle. * 2005 data (adapted from Grave et al., 2010)

BETWEEN-HERD TRANSMISSION DYNAMICS

Current state

Given the high prevalence of LA-MRSA positive pig herds (68%) and the increase in prevalence of positive herds over time *(Chapter 2.3)* it is clear that transmission between herds in The Netherlands does occur. The risk of introduction depends on several factors, e.g. bio-security policies and purchase of animals. Our risk factor analysis *(Chapter 2.3)* was designed to identify and quantify these risk factors. Larger herds were more often positive than smaller herds; the larger the herd, the higher the probability to be MRSA positive, which might be due to the higher risk of introduction into larger herds (between-herd dynamics) or a higher probability of persistence in larger herds (within-herd dynamics) (Gardner et al., 2002).
Differences in herd management associated with herd size might contribute to the identified effect of herd size. Our study showed a significant association between several management variables (e.g. purchase of gilts, hygiene score and antimicrobial use) and herd size, whereas the effect of each individual management variable was too small to yield a significant effect on prevalence of LA-MRSA positive herds.
Introduction of LA-MRSA into a herd can take place through different transmission routes. Airborne transmission of LA-MRSA between herds might play a role in transmission when herds are in close proximity. Antibiotic-resistant *S. aureus* and other multidrug resistant organisms were recovered outside pig facilities to at least 150 m downwind (Gibbs et al., 2006). The relative contribution of this route needs further investigation. The finding of LA-MRSA in black rats *(Rattus rattus)* captured at pig farms suggests that rats might play a role in the transmission of LA-MRSA between pig farms as well (Van De Giessen et al., 2009). Rodents seem to play a role in the transmission within and between pig and poultry farms of other zoonotic bacteria, such as *Salmonella* and *Campylobacter* spp. (Davies and Wray, 1995; Meerburg et al., 2006). It is very likely that this is applicable for LA-MRSA as well. Furthermore, LA-MRSA might be introduced by visitors, feed or materials entering the pig units (Amass et al., 2006).
Another transmission route for LA-MRSA between herds is transmission through the pig production chain, as shown in *Chapter 2.2*. Herds purchasing pigs from LA-MRSA positive herds were more likely to be LA-MRSA positive than herds purchasing pigs from LA-MRSA negative herds. In this study, the relatedness of MRSA isolates within one chain could not be confirmed by the typing method used, i.e. *spa* typing, due to low numbers of isolates and the high frequency of *spa* type t011 and t108. Therefore, isolates from 6 chains our of our study *(Chapter 2.2)* were additionally typed by Pulsed Field Gel Electrophoresis (PFGE) using *Cfr9*I (Bosch et al., 2010). In 4 of the 6 chains (Chain F, P, Q and R), the isolates clustered together in unique PGFE-profiles (Fig. 4). Most notable was that the 6 isolates from Chain F had indistinguishable PFGE-profiles, whereas the isolates belonged to 3 different spa types (t108, t943

and t2503). This molecular association between strains within a pig production chain confirms that transmission of LA-MRSA takes place by animal trade.

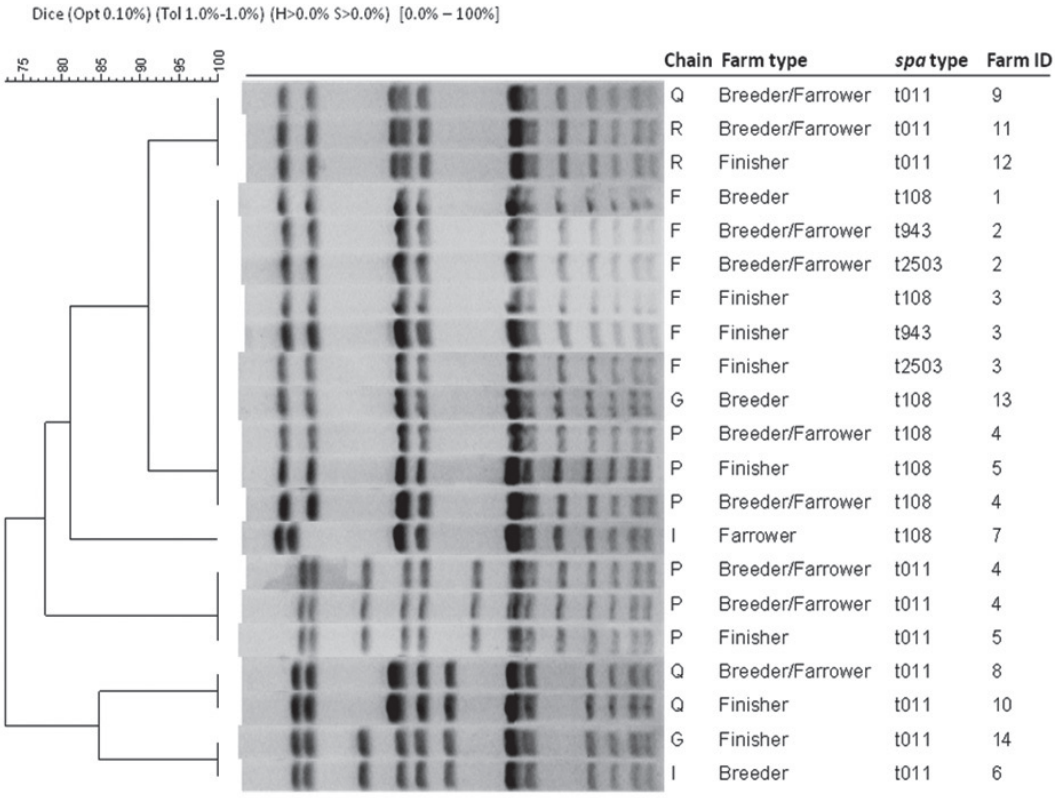


Figure 4. Genetic diversity of 21 MRSA CC398 isolates from 6 pig farms at the end of the production chain (Finisher) and their corresponding gilt or pig suppliers (Breeder, Farrower of mixed Breeder/Farrower) in The Netherlands, with their production chain, farm type, spa type and farm number.

INTERVENTION

The research in this thesis has contributed to a better understanding of the transmission routes of LA-MRSA between pig herds and risk factors for pig herds to be positive. Nevertheless, we should be aware that still not all routes and their contribution to the introduction of LA-MRSA into a herd are elucidated. A large risk was associated with purchasing pigs from MRSA positive herds, but the finding of MRSA positive herds lacking this risk factor, clearly indicates that other risk factors play a role as well.

Farms in close proximity of LA-MRSA positive farms might be at risk of becoming positive by airborne transmission, so in the infrastructure of agricultural regions it might be recommendable to consider minimal distances between farms. Air filtration systems, used in some modern farming systems, might also

contribute in reducing the emission and invasion of pathogens (Dee et al., 2010a, 2010b). Strict rodent control should be a part of biosecurity protocols to reduce the transmission of pathogens through that route. Furthermore, external biosecurity protocols for persons should be optimal, preventing introduction by visitors, feed and materials.
Given the important transmission route through animal trade, a top-down strategy is expected to generate the best gain. Especially for sow herds, it will be feasible to keep the herd closed and be self-supplying for new breeding animals.
A national program to control LA-MRSA starting with eradication of LA-MRSA from breeding herds, followed by a top-down strategy along the pig production chain might be a promising strategy and will be required to be successful for the entire pig sector. Potential intervention measures for the reduction or eradication of LA-MRSA in breeding herds (within-herd dynamics) will be discussed below.

WITHIN-HERD TRANSMISSION DYNAMICS

Current state

Within-herd transmission of LA-MRSA, which can survive for long periods outside the host (Quinn et al., 2002), can occur through many possible transmission routes: through direct contact between animals within a pen or between pens and through indirect contact via persons, equipment, the environment, rodents or air. LA-MRSA does not appear to cause disease or induce life-long immunity in pigs *(Chapter 4.1)*, and therefore a Susceptible-Infectious-Susceptible *(SIS)*-model can be used to quantify transmission of LA-MRSA. *SIS*-models imply that an infection can persist within the population and can become endemic for a long period of time. Whether or not LA-MRSA becomes endemic after introduction into a herd, can be determined by the reproduction ratio, R_0. If $R_0 > 1$, an endemic equilibrium will be established where the proportion of positive individuals is given by $1 - 1/R_0$ (Allen and Burgin, 2000).
Rapid transmission of LA-MRSA within pig herds is facilitated by high contact rates between animals, poor internal biosecurity and animal movements from one pen or section to another. Transmission rates of LA-MRSA were estimated under experimental conditions *(Chapter 4.1)*. The R_0 for LA-MRSA was estimated to be ~4, which indicates that a typical infectious pig will infect on average 4 other pigs during its entire infectious period when introduced into a fully susceptible population. The estimated R_0 implies that the proportion of infectious pigs at an endemic situation is about 75%. The survival of LA-MRSA in the environment implies a much longer 'infectious period of the environment' than the average length of the infectious period observed in the transmission experiment *(Chapter 4.1)*. An infectious period is only valid for infectious individuals. How the contamination of the environment reflects the prevalence of pigs in this environment, how long LA-MRSA persists in the environment, and how environmental contamination affects transmission, is unknown. An *SIS*-model is, therefore, not applicable in situations where no or very few pigs are LA-MRSA positive in a

section, but where LA-MRSA is only present in the environment.
In a longitudinal field study *(Chapter 4.2)*, transmission rates varied largely, but on all farms, LA-MRSA was found at all sampling moments during the study period of ~6 months, indicating persistence within the herd. The transmission rates of LA-MRSA depended on the age of the pigs, the source of infection and on antimicrobial use. In pre-weaning pigs, transmission rates were higher than in post-weaning pigs. This might be explained by the presence of the sow as a source of infection, by a higher susceptibility of newborn piglets or by a greater impact of antimicrobials on unbalanced microbiota in new born piglets (Zoetendal et al., 2004b; Bailey et al., 2005). Transmission rates increased with an increasing relative proportion of positive pen mates compared to the total infection pressure, which includes positive pigs in other pens in the same section and positive environment. So, direct contact between pigs seems to play an important role in the transmission of LA-MRSA. Use of risk antimicrobials, i.e. tetracyclins and β-lactams[1], increased the transmission rates which might be explained by the selective advantage of LA-MRSA compared to susceptible bacteria when these antimicrobials are used (Kadlec et al., 2009). A study on veal farms showed that batch-treated calves were more often LA-MRSA positive than untreated calves (Graveland et al., 2010). Also in humans, the association between antimicrobial use and the occurrence of MRSA was shown (Beam and Buckley, 2006; Muller et al., 2006; Dancer, 2008). The same association was suggested for pig husbandry (De Neeling et al., 2007; Van Duijkeren et al., 2008), but appeared to be hard to confirm in field studies. No significant association was found between antimicrobial use and LA-MRSA in our risk factor analysis for LA-MRSA in sow herds *(Chapter 2.3)*. Most farms in this study did use antimicrobials to some extent and antimicrobial use was associated with herd size, making it hard to untangle the sole effect of antimicrobial use on prevalence of LA-MRSA. Moreover, our transmission experiment proved that LA-MRSA spreads easily between pigs, even without the use of antimicrobials *(Chapter 4.1)*. This was also shown in a longitudinal study on an antimicrobial-free pig farm in Canada where high MRSA prevalence and transmission rates were shown (Weese et al., 2011). On the other hand, in our longitudinal study in pig herds *(Chapter 4.2)* significant higher transmission rates were found when risk antimicrobials were used. This indicates that these antimicrobials positively affect the transmission of LA-MRSA.

INTERVENTION

As stated above, transmission of LA-MRSA and with that persistence within a pig herd can occur through direct or indirect transmission. To reduce transmission through direct contact, the probability of transmission per contact between a susceptible animal and an infectious animal and/or the number of contacts per unit of time should be reduced.
The probability of transmission per contact between animals can be reduced by reducing the number of infectious individuals, shortening the length of the infectious period or reducing the level of shedding of an infectious individual or

[1] Tetracyclins and β-lactams were defined as 'risk antimicrobials', as these antimicrobial classes select 100% for LA-MRSA (Kadlec et al., 2009).

by inducing immunity against infection in susceptible individuals. A first option to reduce the numbers of bacteria shed by infectious individuals and the length of the infectious period of these individuals might be vaccination (Heldens et al., 2001; Potter et al., 2004; Vangeel et al., 2007; Gonzalez et al., 2010). Besides, vaccination might also induce immunity in the susceptible individual against infection and/or disease. As LA-MRSA does not appear to cause disease or induce life-long immunity in pigs, it might be hard to induce immunity by vaccination. After many years of staphylococcal vaccine research for humans and cattle still no effective vaccine has been marketed.

A second option to reduce the number of infectious individuals might be the use of competitive micro-organisms. Interference between strains of the same or different bacterial species might raise resistance to colonization with other strains, such as LA-MRSA. To establish itself in the host, LA-MRSA must successfully compete with many co-existent micro-organisms (Frank et al., 2010). Methicillin susceptible and resistant *S. aureus* strains seem to compete for colonization space in the anterior nares of humans (Dall'Antonia et al., 2005). Other bacteria, such as *Corynebacterium* spp. and *S. epidermidis*, seem to interfere with *S. aureus* colonization as well (Lina et al., 2003). More research is needed to elucidate underlying mechanisms of interactions between bacterial species in pigs and between bacteria and the host.

A third option to reduce the number of infectious individuals and/or the number of bacteria shed by these individuals is reducing antimicrobial use. As the use of antimicrobials selects for resistant strains, including LA-MRSA, a reduction in antimicrobial use might result in a decrease of the within-herd prevalence. A reduction in antimicrobial use might benefit susceptible strains, because antimicrobial resistance might be associated with reduced bacterial fitness (Andersson and Levin, 1999). When selective pressure of antimicrobials is absent, susceptible strains might be able to outcompete resistant strains over time. Nevertheless, studies on this topic showed that the rate of reversibility, i.e. losing resistance genes or outcompetition of resistant strains by susceptible strains, after reduction in antimicrobial use is slow or non-existent, probably due to compensation of fitness costs by so-called compensatory mutations or genes in resistant strains (Austin et al., 1999; Enne et al., 2001; Schulz zur Wiesch et al., 2010; Sundqvist et al., 2010). Knowledge on the bacterial fitness of susceptible and resistant *S. aureus* strains in pigs, and reversibility of antimicrobial resistance in the presence and absence of the selective pressure of antimicrobials, is limited and needs further investigation.

To reduce transmission through indirect contact, the probability of transmission between pens and between sections should be reduced. Groups of pigs, e.g. a litter, should be epidemiologically isolated from other groups as much as possible. This might be achieved by e.g. keeping closed groups, using closed fencing, and extra hygiene precautions, such as cleaning and disinfection, rodent control, using separate clothing for different groups and implementing all-in all-out protocols. Air filtration systems for internal use might also contribute in reducing the emission and transmission of LA-MRSA (Dee et al., 2010a, 2010b).

The experimental design as described in *Chapter 4.1* can be used to quantify the effect of each individual intervention measure mentioned above on transmission rates. Additionally, simulation models can be used to evaluate and identify optimal control strategies (Turner et al., 2006). Probably, a combination of different intervention strategies will be needed to bring transmission below the threshold-value in order to eliminate LA-MRSA from pig farms.

TRANSMISSION FROM PIGS TO HUMANS

Current state

People in contact with pigs, poultry and veal calves are more often LA-MRSA positive than other people, and the intensity of contact is associated with prevalence in these people (Van Den Broek et al., 2009; Graveland et al., 2010; Mulders et al., 2010). This was also shown for pig and poultry slaughterhouse workers and for veterinarians (*Chapter 3.2*; Mulders et al., 2010). In more detail, an overall LA-MRSA prevalence of 14% was found in pig farmers and their family members, limited to persons working or living on positive pig farms. People with intensive contact with positive pigs were more often LA-MRSA positive than people just living on the premises, 49% and 3%, respectively (Van Den Broek et al., 2009). Studies on Dutch veal farms resulted in similar figures: MRSA prevalence in veal farmers and family members was 33% and 8%, respectively, and an increase in prevalence with increasing time spent with animals was shown (Graveland et al., 2010). Both, in pig and poultry slaughterhouse workers, LA-MRSA prevalence was 6% (*Chapter 3.2*; Mulders et al., 2010). Again, contact with live animals was associated with higher LA-MRSA prevalence. This was most obvious in pig slaughterhouse workers, where LA-MRSA was solely found in people working with live animals *(Chapter 3.2)*, In poultry slaughterhouse workers, prevalence in people working with live animals was 5% versus 2% in other personnel (Mulders et al., 2010).
Above mentioned figures originate from single nasal samples, so conclusions whether the isolation of LA-MRSA from these samples are a result of real colonization with LA-MRSA or repeated contamination due to inhalation of dust cannot be drawn. It is essential to distinguish between contamination and colonization, as this will have important consequences for control strategies specific for LA-MRSA. Based on limited studies, the proportion of long term carriers among hospital-acquired MRSA strains seems to vary between 10 to 20% (Marschall and Muhlemann, 2006; Robicsek et al., 2009). Results of 2 Dutch studies indicate that LA-MRSA is strongly associated with recent and repeated exposure to positive pigs or veal calves (Graveland et al., 2011; Van Cleef et al., 2011a). Seventeen percent of field workers taking samples on pig and veal farms tested MRSA positive directly after the farm visit, but 24h later, 94% of them tested MRSA negative again, indicating short term contamination (Van Cleef et al., 2011a). Another study among veal farmers and their family members confirmed this: during holidays, i.e. no contact with veal calves MRSA prevalence was

reduced from 26% to 11%, and only 7% of the participants appeared to be persistent carriers (Graveland et al., 2011). The findings suggest that LA-MRSA is a poor colonizer of humans, and that isolation of LA-MRSA from nasal samples of these persons is most likely a result of repeated contamination and not real colonization.

Prevalence of MRSA in the Dutch general population is estimated below 1% (Wertheim et al., 2004a; Donker et al., 2009), whereas in the population of household members of pig and veal farmers, prevalence is 3% and 8%, respectively (Van Den Broek et al., 2009; Graveland et al., 2010). The isolation of LA-MRSA in household members of pig and veal farmers might be due to animal-to-human transmission through direct contact, when these persons do enter the premises now and then, or from environmental or airborne transmission. Another possibility is human-to-human transmission (discussed below).

INTERVENTION

Reducing the intensity of contact with livestock seems the most successful intervention measure to take, as this is the most important risk factor for people to be MRSA positive. Household members might be able to avoid contact with animals as much as possible, but for people who are professionally in contact with livestock, such as farmers and veterinarians, this will not be feasible. The intensity of contact might be reduced by wearing protective clothing and strict hand hygiene, which are commonly used intervention measures to reduce transmission of MRSA in hospitals (Kappstein et al., 2009). Using masks and gloves might be protective for people who have occupational contact with animals as well. Results of studies on the effect of protective clothing are inconclusive. LA-MRSA can be found in masks of farmers and veterinarians after wearing, confirming airborne exposure within farms (Nathaus et al., 2011; Broens et al., unpublished data). A longitudinal study in 7 German vets, indicated a protective effect of wearing masks (Nathaus et al., 2011), whereas in an international study among veterinarians, wearing a mask was not protective (Wulf et al., 2008b). Further studies are needed to quantify the protective effect of masks, and other protective clothing.

Another potential infection control strategy might be decolonization of MRSA positive persons, which has been shown to be successful in hospitalized patients, both over the short and long term (Dow et al., 2010). However, the expected success rate of decolonization in people who are recurrently in contact with the source of LA-MRSA is very low. One of the typical characteristics of initial patients indicating an association between pig farming and LA-MRSA, was the repeated failure of decolonization therapy, probably due to repeated contamination (Voss et al., 2005).

Eliminating, or at least reducing, the source of LA-MRSA seems the best way to go. When the prevalence of LA-MRSA within the herd is reduced, with that the transmission rate of LA-MRSA from pigs to humans will be reduced.

TRANSMISSION BETWEEN HUMANS

Current state

The Dutch surveillance system for MRSA is used to monitor and check the national search-and-destroy policy. This policy implies screening of high risk patients at hospital admission ('search'). High risk patients are known carriers of MRSA, patients repatriated from a foreign hospital or a Dutch hospital with an on-going MRSA outbreak, patients in close contact with a MRSA carrier, and people in contact with live pigs or veal calves (the latter were assigned to the high risk group in 2006). All identified carriers of MRSA are strictly isolated during hospitalization and treatment is started to eliminate colonization ('destroy'; WIP, 2007). An isolate from all index-cases of MRSA are sent to the National Institute for Public Health and the Environment for genotyping. This enables epidemiological investigation and classification of isolates into hospital-associated (HA), community-associated (CA) and livestock-associated (LA) strains. The first livestock-associated strain was found, retrospectively, in 2003. An increasing proportion of LA-MRSA was seen since then up to 42% of all MRSA isolates in 2008 and 2009 (Haenen et al., 2010; Fig. 5).

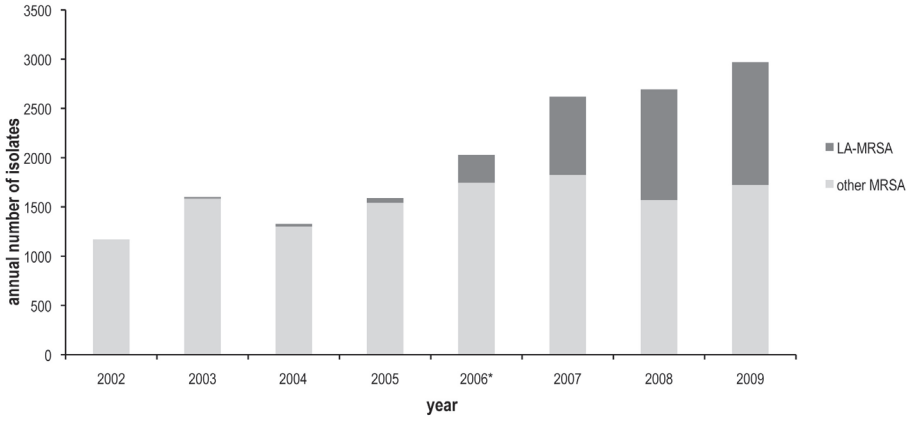


Figure 5. Annual number of LA- and other MRSA isolates from 2002 to 2009 in The Netherlands. *In 2006, people in contact with live pigs and calves were added to the high risk category for screening at hospital admission (adapted from Haenen et al., 2010).

An increased awareness and the active screening of people in contact with live pigs and veal calves since 2006 has obviously contributed to this increase, confirmed by the relatively low proportion of clinical isolates among LA-MRSA compared to other MRSA strains (Haenen et al., 2010). A European survey on the proportion of LA-MRSA of all MRSA isolates showed proportions ranging from 0 to 25%. Also in Europe, the proportion of LA-MRSA was much lower among

clinical isolates (Van Cleef et al., 2011b).
The relatively low proportion of LA-MRSA among clinical isolates might suggest a lower virulence of LA-MRSA compared to other MRSA strains, which was confirmed by a limited study in a Dutch hospital (Van Rijen et al., 2008). Only 13% of patients with a LA-MRSA had an active infection compared to 42% of patients with a HA-MRSA. Nevertheless, the high proportion of LA-MRSA among MRSA isolates (Fig. 5) and the high prevalence of LA-MRSA among people in contact with live pigs and veal calves implies a higher introduction rate of LA-MRSA into hospitals compared to HA-MRSA, especially in areas with high livestock density (Wulf et al., 2011). Outbreaks of LA-MRSA in health-care settings have indeed been reported several times (Wulf et al., 2008a; Fanoy et al., 2009) and invasive infections do occur, not only in people in contact with livestock (Ekkelenkamp et al., 2006; Declercq et al., 2008; Hartmeyer et al., 2010; Mammina et al., 2010; Wulf et al., 2011).
Whole genome analysis of a LA-MRSA isolate from a case of human endocarditis showed considerable differences relative to other *S. aureus* sequences (Schijffelen et al., 2010). Multiple different antimicrobial resistance elements were identified, but most well-known virulence factors for *S. aureus*, such a enterotoxins and Panton-Valentine-Leukocidin (PVL), were lacking. However, an enhanced ability to acquire mobile elements was suggested, which might result in the uptake of mobile elements encoding for virulence genes. This is illustrated by publications on PVL-positive LA-MRSA isolates causing invasive infections in people (Welinder-Olsson et al., 2008; Rasigade et al., 2010). Additionally, the heterogeneity of antimicrobial resistance profiles found in LA-MRSA isolates underlines the ability of LA-MRSA to acquire multiple antimicrobial resistance genes (Kadlec et al., 2009; Argudin et al., 2011; Wulf et al., 2011).
The number of secondary cases among contact persons in a Dutch hospital was found to be 4 times lower for LA-MRSA compared to HA-MRSA, resulting in a relative transmission risk for LA-MRSA of 0.28 (95% CI: 0.09-0.90) compared to HA-MRSA (Wassenberg et al., 2011). The transmissibility of LA-MRSA between humans was also estimated using mathematical modelling. Nosocomial transmission rates were determined for LA-MRSA and other HA-MRSA isolates using observational data from MRSA outbreaks in Dutch hospitals. Based on these data, LA-MRSA appeared to be 6 times less transmissible than HA-MRSA (Bootsma et al., 2011). The reproduction ratio for LA-MRSA was estimated to be significantly below 1, indicating that introduction of LA-MRSA into a hospital will most probably not lead to major outbreaks and an endemic situation.

INTERVENTION

The search-and-destroy policy for MRSA in The Netherlands has proven to be successful in keeping MRSA prevalence low in the general population and in hospitals (Wertheim et al., 2004a). The increase in the annual number of MRSA index cases in The Netherlands, predominantly caused by LA-MRSA, implies an increase in work load and costs for MRSA screening among the newly identified high

risk group ('search' part of the policy). The 'destroy' part of the policy encounters another problem, already indicated in the paragraph on transmission from pigs to humans. Decolonization of people recurrently in contact with livestock will not be successful over the long term as long as people return to the source.
Considering the apparently lower virulence and transmissibility of LA-MRSA, one might propose less stringent control measures for LA-MRSA compared to other MRSA strains. In that case, rapid diagnostic tests distinguishing LA-strains from other strains are needed. This might be problematic, as these tests are validated for HA-strains and not for LA-strains. Test performance might be different, potentially lower, for LA-strains (Wassenberg et al., 2010; Giotis et al., 2011). The ability of LA-MRSA to acquire mobile elements encoding for e.g. virulence and resistance genes suggests potential changes to more virulent strains of LA-MRSA. Opinions among infection control experts on adjustment of MRSA guidelines are diversified.
Again, the best way to go seems elimination of the source of LA-MRSA. When the prevalence of LA-MRSA in livestock is reduced, with that the transmission rate of LA-MRSA from livestock to humans and, subsequently, between humans will be reduced.

CHALLENGES TOWARDS A SOLUTION

The key to limiting LA-MRSA transmission from pigs to humans is to eliminate the source, i.e. eradicate LA-MRSA from pig farms. The perfect way to 'freedom of disease' is not elucidated yet, but a combination of different intervention strategies will be needed. Effects of suggested intervention strategies need to be assessed in experimental and field studies. Mathematical modelling will help to understand underlying mechanisms that affect the transmission dynamics of LA-MRSA within and between farms.
The identification of livestock as a zoonotic reservoir for LA-MRSA, and more recent also for Extended Spectrum Bèta Lactamase (ESBL) producing *Enterobacteriacea* (Leverstein-Van Hall et al., 2011), has brought focus into the disproportional use of antimicrobials in veterinary medicine. Despite essential research to get more insight into ways to eradicate LA-MRSA on pig farms, prompt action is needed to prevent further selection for and transmission of LA-MRSA, ESBL-producing bacteria and other resistant organisms. An integrated approach is needed to reduce the use of antimicrobials substantially. Input from several national and international parties is needed for this. Not only the livestock sector, including farmers, veterinarians and food producers, but also (inter)national policy makers, retailers and consumers have a share in the solution.
Antimicrobial use has been an effective and cheap measure for infection control on farms, whereas other measures to control infectious diseases, such as renovation, better feed quality or vaccination, are much more expensive. To enforce compliance, standards and sanctions should be determined by policy makers, preferably on an international level. Banning certain antimicrobial classes for veterinary use is another part of the solution which acquires harmonization on at least

European level. Subsequently, retailers should take responsibility for their products. The free market system enables retailers to import meat from other countries with less stringent policies, which is contradictory and implies unfair competition. Finally, consumers should realize that a sustainable livestock industry has its price. \

MAIN CONCLUSIONS OF THIS THESIS

The research in this thesis has gained more insight into the prevalence and risk factors of LA-MRSA of pig herds, and contributed to a better understanding of the transmission dynamics of LA-MRSA between pig herds, within pig herds and between pigs and humans. The following main conclusions can be drawn from this thesis:

- LA-MRSA is endemic in the Dutch pig population and both within- and between-herd transmission contribute to this situation.
- Animal trade is an important transmission route for LA-MRSA between herds.
- Larger herds are more often MRSA positive than smaller herds.
- LA-MRSA is easily transmitted between pigs and is able to persist in a pig population, even without antimicrobial use.
- Within-herd transmission occurs both through direct contact and environmental contamination.
- The extensive use of antimicrobials in pig husbandry might have resulted in the selection for LA-MRSA, but reduction of antimicrobial use will be insufficient to eliminate LA-MRSA from the pig population.
- Working with live pigs is the most important risk factor for pig slaughterhouse workers to become positive for LA-MRSA; working with dead pigs does not seem to be a risk factor.
- The key to limiting LA-MRSA transmission from pigs to humans is to eliminate the source, i.e. eradicate LA-MRSA from pig farms.

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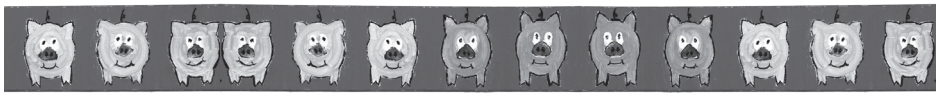

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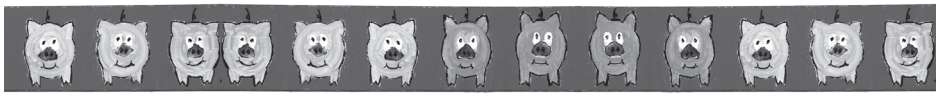
SUMMARY



INTRODUCTION TO LIVESTOCK-ASSOCIATED (LA-)MRSA

Antimicrobial resistance is a public health issue of growing concern. The use of antimicrobials in human and veterinary medicine can lead to development of antimicrobial resistance in micro-organisms. In The Netherlands, antimicrobial use in human medicine is among the lowest, whereas antimicrobial use in veterinary medicine is among the highest in Europe. This high usage of antimicrobials in livestock can result in selection and transmission of resistant bacteria in both pathogenic and commensal bacteria. Transmission of resistant bacteria or their resistance genes from livestock to humans can occur not only by direct contact between animals and humans, but also through the environment, e.g. air or manure, and through food products of animal origin. If these resistant bacteria are zoonotic pathogens, this can directly lead to human disease with potential treatment failure.

Evidence for resistant bacterial infections in humans as a consequence of antimicrobial use in livestock was sparse. However, in 2004 an association between human carriage of methicillin resistant *Staphylococcus aureus* (MRSA) and contact with pigs was identified. Traditionally, MRSA is a human pathogen causing infections and outbreaks in hospitalized patients. Pilot studies confirmed the association between contact MRSA positive people and contact with livestock. Livestock-associated (LA) strains appeared to be not typable by Pulsed Field Gel Electrophoresis (PFGE) with restriction endonuclease SmaI (Dutch standard method), and all strains belonged to Clonal Complex (CC) 398. It was concluded that livestock was a new zoonotic reservoir for MRSA and several research questions about the implications of this finding for public health were raised. More insight into the population dynamics of this LA-MRSA was needed to assess the implications for veterinary and public health. In order to identify (cost-)effective intervention strategies in livestock, and with that to prevent the occurrence of infection in humans, it was essential to gain more insight in transmission dynamics by studying the transmission routes and rates between animals, from animals to humans and from humans to humans as well.

The research described in this thesis aimed to gain more insight in the occurrence and transmission dynamics of LA-MRSA in pig populations. Field and experimental studies were conducted to answer key questions on (1) prevalence of LA-MRSA on different kinds of pig farms, (2) risk factors for introduction and persistence of LA-MRSA in pig populations and the pig production chain, (3) transmission between pigs within a herd as well as transmission between herds, and (4) the role of pig populations in the transmission to humans.

OBSERVATIONAL STUDIES ON LA-MRSA IN THE PIG PRODUCTION CHAIN

First, observational studies are needed to determine the scale of the problem, i.e. estimate the prevalence of MRSA positive herds, and to identify factors associated with LA-MRSA in pig herds. Diagnostic tools are essential in observational studies. In *Chapter 2.1* we compared several sampling methods for the detection

of LA-MRSA in pig herds. Therefore, nasal swabs of pigs and environmental wipes were collected from 147 herds with breeding pigs. Subsequently, nasal swabs were examined in 10 pools of 6 swabs per pool and environmental samples were examined individually and in pools (5 wipes per pool). Large differences in apparent prevalence of MRSA positive herds were found, varying between 19% with pooled wipes to 71% with use of nasal swabs. It was shown that pooling of environmental samples substantially reduced the sensitivity of classifying herds positive. Risk factor analysis based on pooled environmental wipes would, therefore, be questionable. Nevertheless, environmental wipes can be used as an easy and non-invasive method to classify herds for LA-MRSA, but should be individually analysed. The number of wipes needed depends on required detection limits and within-herd prevalences.

A combination of 10 pools of pig samples (6 nasal swabs per pool) and 5 environmental samples, individually analysed, was subsequently used in our observational field studies on herd level. To investigate potential transmission routes for LA-MRSA between herds, a study was performed classifying herds belonging to the same chain within the pig production pyramid *(Chapter 2.2)*. The structure of the Dutch pig production chain enables pathogens to spread between herds during animal trading. Seventy-nine percent of herds with a LA-MRSA positive supplier of pigs were positive, whereas 23% of herds with a LA-MRSA negative supplier were positive. The presence of entirely LA-MRSA positive and -negative chains and the strong association between the LA-MRSA status of herds and their suppliers illustrated a large risk associated with purchasing pigs from LA-MRSA positive herds. A top-down strategy for future control programs is, therefore, a basic requirement. However, the finding of LA-MRSA in herds without any supplier and in herds with a LA-MRSA negative supplier indicated that there are additional risk factors for herds to be LA-MRSA positive.

To determine a national prevalence for LA-MRSA positive pig herds and to identify and quantify risk factors for pig herds to be LA-MRSA positive, an observational study in 31 finishing herds and 171 pig breeding herds was performed *(Chapter 2.3)*. It was shown that LA-MRSA was present in the majority, i.e. ~70%, of Dutch pig herds and that the prevalence increased during the study period from ~30% at the start to ~75% at the end of the study. The prevalence also increased with herd size from < 50% in herds with less than 250 sows to > 75% in herds with more than 500 sows. Other risk factors (e.g. antimicrobial use, purchase of gilts and hygiene measures) were not significantly associated with LA-MRSA in a herd, though these factors were associated with herd size. Herd size appeared to be a compilation of several factors, which made larger herds more often LA-MRSA positive.

It was concluded that LA-MRSA has become endemic in the Dutch pig population and both within and between-herd transmission seem to contribute to this situation. Especially, animal trade is an important transmission route for LA-MRSA between herds.

LA-MRSA IN THE SLAUGHTERHOUSE

A Dutch study in pigs sampled at slaughterhouses showed a much higher prevalence of LA-MRSA than a study where pigs were sampled on farms. The higher prevalence of LA-MRSA in pigs in slaughterhouses compared to the prevalence on farms might be due to LA-MRSA transmission at the slaughterhouse or during transportation to the slaughterhouse. To investigate whether LA-MRSA negative pigs can become positive during transportation from the farm to the slaughterhouse or during waiting time in the slaughterhouse, we sampled 120 pigs from four different LA-MRSA negative farms during loading at the farm, on arrival at the slaughterhouse and after stunning *(Chapter 3.1)*. A clear increase in LA-MRSA positive pigs from 0 to 60% was shown in the few hours between loading and stunning, indicating very rapid transmission of LA-MRSA between pigs through direct contact or through contact with a contaminated environment.
The high prevalence of LA-MRSA in pigs at slaughterhouses, in retail meat and in people working with pigs raises the question whether slaughterhouse workers, who are in contact with pigs (dead or alive) and meat products, are also at risk. Therefore, we performed a study in three Dutch pig slaughterhouses on LA-MRSA in pig slaughterhouse workers, and on the occurrence of LA-MRSA in different slaughterhouse sections during the working day *(Chapter 3.2)*. LA-MRSA was found in nasal swabs from 6% of the pig slaughterhouse workers and working with live pigs was the single most important factor for being LA-MRSA positive. At the start of the day, LA-MRSA was only found in environmental samples taken in the dirty area of the slaughterhouse, whereas at the end of the day LA-MRSA was found in both dirty and clean areas of the slaughterhouse. Exact transmission routes from animals to humans remain to be elucidated. However, it was concluded that working with live pigs is the most important risk factor for pig slaughterhouse workers to become positive for LA-MRSA; working with dead pigs does not seem to be a risk factor.

TRANSMISSION DYNAMICS OF LA-MRSA IN PIG POPULATIONS

At least 2 requirements within a pig population are needed to enable transmission of LA-MRSA from pigs to humans: (1) LA-MRSA should be transmitted between pigs and (2) LA-MRSA should be able to persist in a pig population for a longer time. Transmission and persistence of LA-MRSA within a pig population contributes to the maintenance of the zoonotic reservoir. Transmission can be expressed with the reproduction ratio *(R_0)*, which is defined as the average number of secondary cased caused by one typical infectious individual during its entire infectious period in a completely susceptible population. R_0 has a threshold value of 1; if $R_0 > 1$, minor and major outbreaks can occur and an endemic situation can be established and maintained, whereas $R_0 < 1$, an infection does not spread and will not become endemic, i.e. the infection will fade out. As knowledge on colonization and transmission of LA-MRSA in pigs was limited, we performed two experiments to colonize pigs and quantify transmission of LA-MRSA between pigs

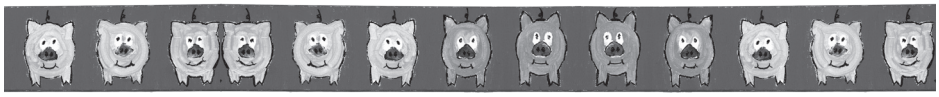
(Chapter 4.1). In the first experiment, colonization of six-week old piglets failed after intranasal inoculation, confirming the complexity of MRSA colonization. In the second experiment, first contact pigs got colonized after exposure to orally inoculated pigs. Transmission rates from first to second contact pigs were determined as well as the length of the infectious period of a colonized pig. A Susceptible-Infectious-Susceptible *(SIS)*-model was used to quantify transmission of LA-MRSA, which implies that an infection can persist within the population and can become endemic for a long period of time. The transmission rate and the length of the infectious period determine the reproduction ratio which was estimated to be around 4 and significantly above 1, indicating a high probability of persistence of LA-MRSA, even without antimicrobial use as these were not applied during the experiment.

Subsequently, a longitudinal field study was performed in Danish and Dutch farrow-to-finish herds to quantify transmission of LA-MRSA and factors affecting it *(Chapter 4.2)*. These herds were classified LA-MRSA positive in earlier studies. Sows and piglets were sampled at varying intervals during a production cycle. Prevalences from sows increased from 33% before farrowing to 77% just before weaning. Prevalences from pigs were > 60% from just after birth until finishing. The recurrent finding of LA-MRSA in the majority of sampled individuals either indicated prolonged or persistent colonization, or might be a result of repeated contamination. Transmission rates were higher when tetracyclins and β-lactams were used, indicating a selective advantage of LA-MRSA when these antimicrobials are used. Furthermore, transmission rates were higher in pre-weaning pigs than in post-weaning pigs, indicating an age-related susceptibility or an effect of the sow. When the infection pressure within the pen increased comparative to the total infection pressure in the section, transmission rates increased, implying that direct contact is an important transmission route. The results of the experimental and longitudinal field study indicate that LA-MRSA is able to spread and persist in a pig population, resulting in an endemic situation.

CHALLENGES TOWARDS A SOLUTION

The extensive use of antimicrobials in pig husbandry might have resulted in the selection for MRSA, but reduction of antimicrobial use will be insufficient to eliminate LA-MRSA from the pig population. The key to limiting LA-MRSA transmission from pigs to humans is to eliminate the source, i.e. eradicate LA-MRSA from pig farms. The perfect way to 'freedom of disease' is not elucidated yet, but a combination of different intervention strategies controlling both between and within-herd transmission will be needed. An integrated approach with input from several national and international parties, including farmers, policy makers, retail, human and veterinary doctors, and consumers, is needed to come to a solution.

SAMENVATTING EN CONCLUSIES



AANLEIDING VOOR DIT ONDERZOEK

De laatste jaren is er een groeiende zorg over de wereldwijde toename van bacteriën die resistent zijn tegen antibiotica. In Nederland is, door een restrictief antibioticabeleid en een zorgvuldig infectiepreventiebeleid in de humane gezondheidszorg, het resistentieprobleem tot op heden beheersbaar gebleven. Jaarlijkse rapportages laten echter een gestage toename van zowel antibioticagebruik als -resistentie in de humane gezondheidszorg zien. Eén van de belangrijkste en bekendste resistente bacteriën is meticilline resistente *Staphylococcus aureus* (MRSA), ook wel 'ziekenhuisbacterie', genoemd. Gezonde personen kunnen MRSA bij zich dragen, meestal in de neus, zonder daar last van te hebben. Onder bepaalde omstandigheden, bijvoorbeeld bij een verminderde afweer of een operatiewond, kan MRSA infecties veroorzaken, die dan lastig behandelbaar zijn door de ongevoeligheid van deze bacterie tegen verschillende soorten antibiotica. MRSA was voorheen een bacterie die zich beperkte tot infecties en uitbraken in ziekenhuizen, en was dan vaak afkomstig van zogenaamde risico-patiënten, waaronder personen die in buitenlandse ziekenhuizen waren verpleegd. In Nederland wordt MRSA in ziekenhuizen onder controle gehouden door strikte richtlijnen die voorschrijven dat personen uit risicogroepen (bv. patiënten die in een buitenlands ziekenhuis verpleegd zijn) gescreend moeten worden op MRSA en, indien positief bevonden, geïsoleerd behandeld worden.

In 2004 werd MRSA aangetoond bij enkele patiënten die niet tot een bekende risicogroep behoorden. De gevonden MRSA-stammen werden voor nader onderzoek naar het RIVM gestuurd, maar bleken niet typeerbaar met de gebruikelijke methode. Een opmerkzame medisch microbioloog ging op zoek naar de overeenkomst tussen deze MRSA-gevallen en vermoedde daarop een verband met varkens. Dit vermoeden werd bevestigd toen bij enkele varkens van een bedrijf waarop één van de positief bevonden personen woonachtig was, ook deze niet-typeerbare MRSA in de neus werd aangetroffen. Vervolgonderzoek bevestigde dat personen die veel in contact waren met varkens, maar ook met vleeskalveren, vaker MRSA bij zich droegen dan andere personen. De ontdekking van varkens, en andere productiedieren, als potentiële bron van MRSA voor mensen, leidde tot een aanpassing van de ziekenhuisrichtlijnen. Personen in contact met levende varkens en vleeskalveren werden toegevoegd tot de risicogroep en dus gescreend voor MRSA bij ziekenhuisopname.

Om beter inzicht te verkrijgen in de omvang van het probleem en de implicaties van deze 'veegerelateerde' MRSA (v-MRSA) voor de humane en veterinaire gezondheidszorg was meer onderzoek noodzakelijk. Het toenmalige ministerie van Landbouw, Natuur en Voedselkwaliteit gaf opdracht tot uitvoering van een uitgebreid onderzoeksprogramma MRSA, waarvan het ministerie van Volksgezondheid, Welzijn en Sport de financiering van de humane onderzoekscomponent op zich nam. Drie projecten binnen dit onderzoeksprogramma die zich toespitsten op de varkenshouderij zijn onderdeel van dit proefschrift *(Hoofdstukken 2.2, 2.3 en 3.1)*. Aanvullend onderzoek is uitgevoerd om inzicht te verkrijgen in de verspreiding van v-MRSA tussen varkens, tussen varkensbedrijven en tussen varkens en mensen.

VEEGERELATEERDE MRSA IN DE VARKENSHOUDERIJ

Verschillende onderzoeken op varkensbedrijven werden uitgevoerd om inzicht te verkrijgen in het vóórkomen van v-MRSA op deze bedrijven en factoren die bepalen waarom bepaalde bedrijven positief zijn en anderen niet. In *hoofdstuk 2.1* zijn eerst verschillende methodes om v-MRSA aan te tonen op een bedrijf met elkaar vergeleken. Op basis van neusswabs afgenomen bij dieren, die werden onderzocht in pools (zes swabs per pool), werd 71% van de bedrijven positief bevonden en op basis van vijf, individueel onderzochte, stofmonsters was dit 53%. Als de vijf stofmonsters in één pool werden onderzocht in het laboratorium was slechts 19% van de bedrijven positief. Het onderzoeken van stofmonsters is een diervriendelijke en makkelijke methode en geeft dus een redelijke indicatie van de v-MRSA status van een varkensbedrijf. Stofmonsters moeten echter niet gepoold worden, omdat dit de gevoeligheid van de test drastisch verlaagd. In vervolgonderzoeken op varkensbedrijven is een combinatie van vijf individueel onderzochte stofmonsters en tien pools van zes neusswabs gebruikt om de v-MRSA status van varkensbedrijven te bepalen.
In de varkenshouderij in Nederland worden gelten vervoerd van fok- naar vermeerderingsbedrijven. Vervolgens worden vleesbiggen vervoerd van vermeerderings- naar vleesvarkensbedrijven. Via deze route kunnen infectieuze organismen, inclusief v-MRSA, vrij makkelijk verspreid worden tussen bedrijven. In het onderzoek beschreven in *hoofdstuk 2.2* is gekeken in hoeverre de handel van dieren tussen varkensbedrijven bijdraagt aan de verspreiding van v-MRSA tussen bedrijven. Bedrijven die varkens aanvoerden van v-MRSA positieve bedrijven waren vaker (79%) zelf ook positief dan bedrijven die varkens aanvoerden van v-MRSA negatieve bedrijven (23%). Handel van dieren speelt dus een belangrijke rol in de verspreiding van v-MRSA tussen bedrijven. Bij de bestrijding van v-MRSA in de varkenshouderij is het dus van belang om bovenaan de keten, bij de fokbedrijven, te beginnen.
Onderzoek op 202 Nederlandse varkensbedrijven *(Hoofdstuk 2.3)* toonde aan dat v-MRSA voorkomt op ~70% van de bedrijven. Het aantal v-MRSA positieve bedrijven nam toe gedurende de onderzoeksperiode van ~30% begin 2007 tot ~75% eind 2008. Bedrijfsgrootte had ook een effect op het vóórkomen van v-MRSA: meer dan 75% van de grote bedrijven (> 500 zeugen) werd positief bevonden, terwijl van de kleinere bedrijven (< 250 zeugen) minder dan 50% positief was. Andere factoren (o.a. antibioticagebruik, aankoop van dieren en hygiënemaatregelen) waren niet geassocieerd met v-MRSA op een bedrijf, maar waren wel geassocieerd met bedrijfsgrootte. De factor bedrijfsgrootte is hiermee een verzameling van allerlei (risico)factoren, die gezamenlijk ervoor zorgen dat grotere bedrijven vaker v-MRSA positief zijn.

VEEGERELATEERDE MRSA IN HET SLACHTHUIS

In 2007 werd in een onderzoek op varkensslachthuizen een onverwacht hoog aantal v-MRSA positieve dieren gevonden in vergelijking met onderzoeken waarbij

varkens op het bedrijf bemonsterd werden. Een mogelijke verklaring voor het gevonden verschil zou verspreiding van v-MRSA tussen varkens tijdens het transport naar het slachthuis of tijdens het verblijf in de wachtruimte van het slachthuis kunnen zijn. Om dit nader te onderzoeken werden 117 dieren afkomstig van vier v-MRSA negatieve bedrijven op drie tijdstippen bemonsterd, namelijk tijdens het opladen op het bedrijf, tijdens het afladen op het slachthuis en op de steektafel (= na verblijf in de wachtruimte) van het slachthuis *(Hoofdstuk 3.1)*. Tijdens het opladen waren alle dieren negatief, terwijl op de steektafel bij 60% van de dieren v-MRSA werd gevonden in de neus. In de tussentijd zijn de varkens dus met v-MRSA besmet geraakt door contact met positieve dieren afkomstig van andere bedrijven of door contact met een besmette omgeving.
Aangezien v-MRSA al eerder werd aangetoond in slachtvarkens, in varkensvlees en bij varkenshouders, is er mogelijk ook een verhoogd risico voor slachthuismedewerkers. Om dit te onderzoeken werden in drie Nederlandse slachthuizen omgevingsmonsters genomen van de verschillende afdelingen van het slachthuis en neusswabs van slachthuismedewerkers *(Hoofdstuk 3.2)*. In de omgevingsmonsters werd aan het begin van de werkdag alleen v-MRSA aangetoond in het vuile gedeelte (= daar waar levende varkens verblijven). Aan het eind van de werkdag, werd v-MRSA ook gevonden in andere gedeeltes van het slachthuis. Bij 6% van de medewerkers werd v-MRSA aangetoond in de neus, en dit bleken allen medewerkers te zijn die met levende varkens in contact kwamen. Onder medewerkers die niet met levende varkens in contact kwamen, werd geen v-MRSA gevonden.

VERSPREIDING VAN VEEGERELATEERDE MRSA

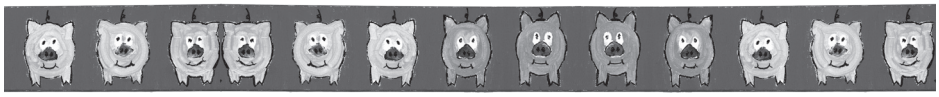
Verspreiding van v-MRSA tussen varkens en blootstelling van mensen aan deze bron van v-MRSA is nodig om overdracht van v-MRSA van varkens naar mensen mogelijk te maken. De mate van verspreiding van v-MRSA op een varkensbedrijf kan berekend en uitgedrukt worden in een getal, de R_0-waarde. De R_0-waarde heeft een drempelwaarde van 1; als $R_0 < 1$, dan kan een infectie zich niet verspreiden en zal uitdoven, als $R_0 > 1$, dan kan een infectie zich wel verspreiden en zal de infectie zich mogelijk handhaven op een bedrijf. Om meer kennis over de verspreiding van v-MRSA tussen varkens te vergaren, werden twee experimentele studies uitgevoerd *(Hoofdstuk 4.1)*. In het 1^e experiment werd bij vijf biggen een suspensie met v-MRSA in de neus toegediend. Bij geen van de dieren werd vervolgens v-MRSA aangetoond. In het 2^e experiment, werd bij vijf biggen een suspensie met v-MRSA oraal toegediend. Deze vijf biggen werden vervolgens bij vijf niet-besmette dieren geplaatst (1^e contact dieren). Nadat bij de 1^e contactdieren v-MRSA was aangetoond werden deze overgeplaatst naar een andere ruimte met opnieuw vijf niet-besmette dieren (2^e contactdieren). De mate van verspreiding tussen 1^e en 2^e contactdieren werd berekend, evenals de lengte van de infectieuze periode (= aantal dagen dat de dieren achtereen positief werden bevonden). Een wiskundig model werd gebruikt voor de berekening waarbij aangenomen werd dat dieren gevoelig of infectieus kunnen zijn, en dat er geen immuniteit optreedt tegen v-MRSA. De R_0-waarde werd geschat op ~4, en dus ruim

boven de drempelwaarde. Dit wijst er op dat v-MRSA zich kan verspreiden en handhaven binnen een groep varkens, zelfs zonder antibioticagebruik, aangezien deze niet werden toegepast tijdens het experiment.
Vervolgens werden op 6 varkensbedrijven, 2 Deense en 4 Nederlandse, varkens bemonsterd om de mate van verspreiding en factoren die daarop van invloed zijn te kunnen bepalen. Zeugen (~10 per bedrijf) en hun biggen werden bemonsterd op verschillende tijdstippen gedurende een productiecyclus. Voor het werpen was 33% van de zeugen v-MRSA positief en dat percentage liep op naar 77% rondom spenen (~4 weken na werpen). Van de biggen was vanaf geboorte tot afleverleeftijd (~6 maanden) telkens meer dan 60% v-MRSA positief. De mate van verspreiding nam toe als bepaalde antibiotica (tetracyclines en β-lactams) werden toegepast, wat wijst op een selectief voordeel voor v-MRSA als deze antibiotica worden gebruikt. Daarnaast was de mate van verspreiding hoger bij biggen vóór het spenen dan bij biggen na het spenen, wat kan wijzen op een leeftijd gerelateerde gevoeligheid of op een invloed van de aanwezigheid van de zeug. Direct contact tussen varkens in hetzelfde hok bleek de belangrijkste route te zijn voor de verspreiding van v-MRSA. De resultaten van de experimenten en van de studie op varkensbedrijven tonen aan dat v-MRSA in staat is om zich te verspreiden en te handhaven op een varkensbedrijf.

BELANGRIJKSTE CONCLUSIES VAN DIT PROEFSCHRIFT

- v-MRSA is alom aanwezig in de Nederlandse varkenshouderij en verspreiding vindt niet alleen plaats tussen varkens binnen hetzelfde bedrijf, maar ook tussen bedrijven.
- Handel in dieren is een belangrijke verspreidingsroute van v-MRSA tussen bedrijven.
- Grote bedrijven zijn vaker v-MRSA positief dan kleine bedrijven.
- v-MRSA kan zich verspreiden en handhaven op een varkensbedrijf, ook als geen antibiotica gebruikt worden.
- v-MRSA wordt binnen een bedrijf verspreid door direct contact tussen varkens en door contact met een besmette omgeving.
- Het op grote schaal toedienen van antibiotica in de varkenshouderij heeft waarschijnlijk geresulteerd in de selectie en verspreiding van v-MRSA, maar terugdringen van het antibioticagebruik is onvoldoende om v-MRSA te elimineren van varkensbedrijven.
- Contact met levende varkens is de belangrijkste risicofactor voor slachthuispersoneel om positief te testen voor v-MRSA; werken met dode varkens blijkt geen risico te vormen.
- Om de overdracht van v-MRSA van varkens naar mensen te beperken moet de bron geëlimineerd worden, oftewel v-MRSA moet op het varkensbedrijf bestreden worden.

ABOUT THE AUTHOR



CURRICULUM VITAE

Elisabeth (Els) Marion Broens was born on April 20th 1972 in Westendorp. In 1989, she graduated from the 'Gemeentelijke Scholen Gemeenschap Doetinchem (GSGD)', and started her study at the Faculty of Veterinary Medicine, Utrecht University. After her graduation in 1998, she worked as a farm animal veterinarian at the 'Veterinair Centrum Aadal' in Heeswijk-Dinther for 1.5 year. In 1999, she returned to the Faculty of Veterinary Medicine and worked as a junior lecturer at the Department of Farm Animal Health, where she focused on clinical diseases of ruminants and dairy herd health management. In 2003, she moved to the Department of Infectious Diseases and Immunology from the same faculty, where she lectured on microbiology and infectious diseases. At the same time, she started her residency to become a Specialist in Veterinary Microbiology. To broaden her horizon and to increase her research skills she started as a project leader in 2006 at the Division of Infectious Diseases of the Animal Sciences Group (nowadays Central Veterinary Institute) in Lelystad. There, she worked on development and validation of diagnostic tests for zoonotic pathogens and was a safety officer for biological agents. In August 2007, she started her PhD research on methicillin resistant *Staphylococcus aureus* in pigs at the Quantitative Veterinary Epidemiology Group of Wageningen University and the Laboratory for Zoonoses and Environmental Microbiology of the Centre for Infectious Disease Control Netherlands at the RIVM in Bilthoven. The veterinary and microbiological aspects of the research project matched her prior interests and experience, whereas the quantitative epidemiological aspects were a challenging, but welcome extension of her expertise. Els hopes to finalize her residency in Veterinary Microbiology, together with the conferral of her doctorate in October 2011. In August 2011, Els returned to the Department of Infectious Diseases and Immunology of the Faculty of Veterinary Medicine, where she will become head of the Veterinary Microbiological Diagnostic Centre (VMDC).

LIST OF PUBLICATIONS

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CONFERENCE PROCEEDINGS AND ABSTRACTS

- Porphyre T, Loeffler A, Lloyd DH, Giotis E, Broens EM, Stärk KDC (2011). Modelling the spread of MRSA ST398 to communities: role of persistent carriage and pig exposure. *Proceedings of 4th symposium on antimicrobial resistance in animals and the environment* (AREA 2011), 27-29 June 2011, Tours, France.

- Van Der Wolf PJ, Veldkamp M, Broens EM, Köck R, Graat EAM (2011). A longitudinal study on the persistence of livestock associated MRSA in swine herds. *Proceedings book of 9th international conference on the epidemiology and control of biological, chemical and physical hazards in pigs and pork* (Safepork 2011), 19 – 22 June 2011, Maastricht.

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- Broens EM, Bosch T, Van De Giessen AW, Huijsdens XW, Graat EAM (2008). Genetic comparison of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* isolated from Dutch pig farms. *Abstract book of ASM Conference on Antimicrobial resistance in zoonotic bacteria and foodborne pathogens,* 15 – 18 June 2008, Copenhagen, Denmark.

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- Broens EM, Graat EAM, Van Der Wolf PJ, Van Den Broek IVF, Tiemersma EW, Van De Giessen AW, De Jong MCM (2008). MRSA in pigs: a new threat to human health !? How many and which pig farms are MRSA-positive? *WIAS Science Day,* 13 March 2008, Wageningen.

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- Broens EM, Graat EAM, De Jong MCM, Meijerink M, Van Den Broek IVF, Van Cleef BAGL, Huijsdens XW, Mevius DJ, Oosterom RAA, Van Der Wolf PJ (2009). Project 8: Prevalentieschatting en risicofactorenanalyse MRSA bij varkens. RIVM report 330224001: Veegerelateerde MRSA: epidemiologie in dierlijke productieketens, transmissie naar de mens en karakterisatie van de kloon, J.A. Wagenaar en A.W. Van De Giessen (ed.), 49-68.

- Broens EM, Graat EAM, De Jong MCM, Lommerse J, Meijerink M, Huijsdens XW, Mevius DJ, Van Der Wolf PJ (2009). Project 12: MRSA ST398 in de varkensproductiepiramide. RIVM report 330224001: Veegerelateerde MRSA: epidemiologie in dierlijke productieketens, transmissie naar de mens en karakterisatie van de kloon, J.A. Wagenaar en A.W. Van De Giessen (ed.), 116-123.

- Broens EM, Graat EAM, De Jong MCM, Huijsdens XW, Mevius DJ, Van Der Wolf PJ (2009). Project 13: MRSA-transmissie tijdens transport en in het slachthuis. RIVM report 330224001: Veegerelateerde MRSA: epidemiologie in dierlijke productieketens, transmissie naar de mens en karakterisatie van de kloon, J.A. Wagenaar en A.W. Van De Giessen (ed.), 124-129.

TRAINING AND SUPERVISION PLAN

The basic package (3.0 ECTS)	year
WIAS Introduction Course	2008
WIAS Course on Ethics and Philosophy in Animal Science	2010

International conferences (3.9 ECTS)	
20th International Veterinary Pig Society Congress, June 2008, Durban, South-Africa	2008
Seminar of the Danish Pig Veterinary Society, Nov 2008, Kolding, Denmark	2008
ASM conference on methicillin-resistant staphylococci in animals: veterinary and public health implications, Sep 2009, London, United Kingdom	2009
2nd International conference on MRSA in animals, Sep 2009, London, United Kingdom	2009
Annual conference from Society for Veterinary Epidemiology and Preventive Medicine, March 2011, Leipzig, Germany	2011

Seminars and workshops (3.0 ECTS)	
Studiedag Dutch Society for Veterinary Epidemiology and Economics, Wageningen	2007
Zoönosensymposium RIVM, Bilthoven	2007
WIAS Science Day, Wageningen	2008
Workshop Infection Dynamics, Utrecht	2008
Zoönosensymposium RIVM, Bilthoven	2008
Workshop 'Zwanger op de boerderij' (moderator), Bilthoven	2008
Symposium ter afsluiting LNV-onderzoeksprogramma MRSA, Amersfoort	2009
WIAS Science Day, Wageningen	2010
Kennismakingsdag CIb/RIVM voor Medisch en Veterinair Microbiologen i.o.	2010
Zoönosensymposium RIVM, Amsterdam	2010

Presentations (11.0 ECTS)	
Poster at WIAS Science Day, Wageningen	2008
Poster and oral presentations at 20th International Veterinary Pig Society Congress, June 2008, Durban, South-Africa	2008
Oral presentation at seminar of the Danish Pig Veterinary Society, Nov 2008, Kolding, Denmark	2008
Poster and oral presentations at ASM conference on methicillin-resistant staphylococci in animals: veterinary and public health implications, Sep 2009, London, United Kingdom	2009
Oral presentation at 2nd International conference on MRSA in animals, Sep 2009, London, United Kingdom	2009
Oral presentation at symposium ter afsluiting LNV-onderzoeksprogramma MRSA, Amersfoort	2009
Oral presentation at WIAS Science Day, Wageningen	2010
Oral presentation at Annual conference from Society for Veterinary Epidemiology and Preventive Medicine, March 2011, Leipzig, Germany	2011

In-depth studies (14.1 ECTS)	
QVE-30306 Quantitative Veterinary Epidemiology	2008
Statistics for the life sciences, WIAS, Wageningen	2008
Advanced statistics course: Design of animal experiments, WIAS, Wageningen	2009
Course on molecular diagnostics, Erasmus MC, Rotterdam	2009
Design and analysis of transmission experiments, EPIZONE, Wageningen	2009
Course in virology, Erasmus MC, Rotterdam	2010
Advanced course Immunology, Infection & Immunity Centre, Utrecht	2011

Professional skills support courses (3.8 ECTS)	
Project management training, ASG, Lelystad	2007
PhD competence assessment, WGS, Wageningen	2008
Techniques for writing and presenting a scientific paper, WGS, Wageningen	2008
Career assessment, WGS, Wageningen	2010
'Communication with the media and the general public', WGS, Wageningen	2010

Research skills training (11.0 ECTS)	
Preparing own PhD research proposal	2007
WIAS Talents & Topics 2011 – programme	2011

Didactic skills training (9.8 ECTS)	
Lecture in Health, Welfare and Management (ADP-30306)	2008-2009
Supervising 5 BSc students and 5 MSc students	2008-2011

Education and training total	**59.6 ECTS**

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