Methicillin-resistant Staphylococcus aureus in Danish production animals

Hansen, Julie Elvekjær

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Methicillin-resistant *Staphylococcus aureus* in Danish production animals

PhD Thesis • Julie Elvekjær Hansen
Name of Department: National Veterinary Institute, Division of Diagnostics & Scientific Advice – Bacteriology & Parasitology, Technical University of Denmark, Kemitorvet, Building 204, DK-2800 Kgs. Lyngby, Denmark.

Author: Julie Elvekjær Hansen, Cand.scient. Biology & biotechnology, ORCID:0000-0003-3885-8929

Title: Methicillin-resistant *Staphylococcus aureus* in Danish production animals

Supervisors: Professor Karl Pedersen, Division of Diagnostics & Scientific Advice – Bacteriology & Parasitology, Technical University of Denmark

Head of Section, PhD Anders Rhod Larsen, Department of Bacteria, Parasites & Fungi, Statens Serum Institute, Denmark

Senior scientist Anne Mette Madsen, National Research Centre for the Working Environment, Denmark

Assessment committee: Associate Professor Anders Folkesson, Division of Diagnostics & Scientific Advice – Bacteriology & Parasitology, Technical University of Denmark

Professor John Elmerdahl Olsen, Section, Veterinary Clinical Microbiology, University of Copenhagen, Denmark

Professor Jaap Wagenaar, Department of Infectious Diseases and Immunology - Clinical Infectiology, University of Utrecht, the Netherlands

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Preface

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First and foremost, I would like to thank my supervisors Karl Pedersen and Anders Rhod Larsen for your guidance, support and the many scientific discussions, which has helped develop me as a scientist. I also would like to thank Anne Mette Madsen for introducing me to the different concepts of air sampling.

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To all the great people at the veterinary institute whom I have had the pleasure of getting to know, thank you for your help and support, and for creating an enjoyable social atmosphere, both on a normal work day and of cause at Wetbar.

Thank you, Lærke Boye Astrup for the priceless conversations on both personal and scientific matters. Additional thanks for proofreading of my thesis and the constructive feedback. I am greatly appreciative of you spending the time, despite your more than full schedule. It has been a pleasure working with you and I look forward to many more conversations.

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Julie Elvekjær Hansen, December 2017.
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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as one of the most common multi-resistant bacteria found in both human and veterinary medicine, and the newly emerged livestock-associated MRSA clonal complex 398 (LA-MRSA CC398) pose a risk of zoonotic infections in humans. An increase in the occurrence of MRSA CC398 positive carriers in Denmark has been observed in the general community, where a proportion of cases occur in people without livestock exposure. This development threatens the national low levels of MRSA in humans and the sustainability of the domestic ‘search-and-destroy’ control policies.

To arrest this development it seems important to contain MRSA CC398 within pig farms, which are the primary reservoir of MRSA CC398, followed by attempts to lower the within farm level of MRSA CC398. Quantitative methods for MRSA CC398 within farms are needed in order to assess the effect of intervention strategies. However, *S. aureus* has on multiple occasions proven difficult to control and has illustrated its ability to adapt to new niches and disseminate with great success to new reservoirs.

This PhD project presents an evaluation of the feasibility to measure MRSA CC398 within pig herds in a quantitative and reproducible manner using swab and air samples, and an assessment of MRSA CC398 loads within different units of the pig production. We found nasal swab samples to be more reproducible than skin swab samples. Further, nasal samples were found to be a better proxy for airborne MRSA CC398 compared to skin samples, however, the correlation was found insufficient to recommend that air samples would be enough for complete quantification of MRSA CC398 within production units. The highest levels of MRSA CC398 within pig farms were seen in the farrowing and weaning unit which means, that the highest risk for farmers to become colonized with MRSA CC398 occurs when working within these two units. Unfortunately, these two units are where the most intense human-to-pig contact occurs which is worrisome, as human carriage are known to play a role in dissemination of MRSA CC398 into naive animal productions.

Focus was consequently turned to investigate the emergence of possible new animal reservoirs in Denmark. We investigated if Danish veal and dairy herds constitute a reservoir of MRSA CC398. Both production lines was found positive for MRSA CC398 in low prevalence, however veal calf was thought merely to be contaminated, whereas indications of dairy herds as a persistent reservoir was observed. The Danish mink production has gained our attention for some time throughout the present
study period, as human cases of MRSA CC398 in people with mink contact, have been seen since 2009 and infections in mink caused by MRSA CC398 was first observed in 2013. Consequently, we wanted to determine the degree of MRSA CC398 positive farms and to identify where the bacterium most often could be detected. A total of one third of the screened farms were found positive for MRSA CC398 with the bacterium most often found on the paws and in the pharynx, which poses a human health hazard to farmers, who risk getting bites and scratches when handling the animals.

Based on results from phylogenetic analysis of isolates from both the cattle and mink production, we suggested a spillover from the pig production to be the primary source of introduction. In mink, results pointed to an introduction via contaminated feed. The introduction into mink has subsequently led to increased carriage and infections in people with contact to mink, observed since 2011.

The results obtained as part of this PhD project, emphasize the importance of lowering the levels of MRSA CC398 within farms to reduce dissemination and emergence of new reservoirs. Further, the results illustrated the need for continued screening of low prevalence MRSA CC398 positive productions and possibly unknown positive productions. This is needed in order to try and take control of the development, emergence and spread of MRSA CC398, with the subsequent goal of preventing the emergence of reservoirs with possible relevance to human health.
Dansk sammendrag

Methicillin resistente *Staphylococcus aureus* (MRSA) er de mest almindeligt forekommende multiresistente bakterier i både human- og veterinærmedicin. Den nyligt opståede *S. aureus* associeret til dyr, kendt som husdyr-MRSA eller LA-MRSA CC398, udgør en risiko for zoonotiske infektioner i mennesker. Gennem en årrække har man set en stigning i antallet af humane tilfælde af samfundsrelateret MRSA, hvoraf en stabil andel af disse tilfælde er fundet i mennesker uden kontakt til husdyr. Denne udvikling truer det lave niveau af MRSA, der generelt ses i det danske samfund, samt den nationale ”search-and-destroy” politik, som føres i kampen mod MRSA.

For at standse denne uheldige udvikling kan det være vigtigt at undgå, at MRSA CC398 undslipper det primære reservoir, den danske svineproduktion, hvorefter man kan fokusere på at sænke niveauet af MRSA CC398 i gårdsdjævel. Det er muligt, at man ved at implementere en eller flere interventionsstrategier kan sænke niveauet af MRSA CC398, men for at kunne følge en given effekt af et interventionsforsøg, er det essentielt, at der findes kvantitative målemetoder til at bestemme niveauet af MRSA CC398. At kontrollere *S. aureus* har dog tidligere vist sig at være udfordrende, da denne fascinerende bakterie gang på gang har bevist sig i stand til at tilpasse sig nye miljøer og som resultat heraf spredes med stor succes til nye reservoirs, både humane og animale.

Som en del af denne ph.d. afhandling præsenteres en evaluering af mulighederne for at måle MRSA CC398 i forskellige led af svineproduktionen på en både kvantitativ og reproducerbar måde. Vi har testet dette med svaberprøver fra dyr, hhv. trynen og huden, samt taget indendørs luftmålinger af MRSA CC398 niveauet i luften. Resultater fra denne undersøgelse viste, at tryneprøver giver mere reproducerbare måleresultater af niveauet af MRSA CC398 på dyr, samt i højere grad reflekterer det niveau, der kan måles i luften holdt i forhold til hudprøver. Korrelationen mellem niveauet, der ses i trynen og i luften, er dog ikke god nok til, at vi vil anbefale, at man ved kun at tage luftpørører kan opnå et fuldstændigt billede af niveauet af MRSA CC398 i en given staldenhed. De kvantitative målinger viste, at niveauet af MRSA CC398 er højest i farestalde samt fravænsningsstalde, hvilket indikerer, at risikoen for, at landmænd erhverver MRSA CC398 ved arbejde i stalden, er højest i netop disse to staldenheder. Denne observation er bekymrende, da det er i fare- og fravænsningsstalde, at den mest intense kontakt til dyrene findes, hvilket er en risikofaktor for at blive bærer af MRSA CC398. Samtidig ved man fra tidligere studier, at mennesker kan spille en vigtig rolle i spredning af MRSA
CC398 uden for staldmiljøet, hvor humane bærere af MRSA CC398 kan medbringe smitte fra et kontamineret til et hidtil MRSA-frit staldmiljø.

Resultatet af denne kvantitative undersøgelse af MRSA CC398 i svin fik os til at vende opmærksomheden mod nyligt opståede, ukendte reservoirs i andre danske produktionsdyr. Vi undersøgte tilstedeværelsen af MRSA CC398 i både dansk kalve- og mælkeproduktion og fandt, at den findes i begge produktionssystemer, men i en lav grad. Der var indikationer på, at tilstedeværelsen i kalve muligvis skyltes kontamination, mens tilstedeværelsen i mælkeproduktionen så ud til at kunne være persistender. En anden stor dansk produktion af mulig interesse var produktionen af mink, da man siden 2009 har set humane tilfælde af infektioner med MRSA CC398 hos minavlere, mens man i 2013 fandt MRSA CC398 infektioner i to mink, der var indsendt til diagnostisk undersøgelse på det DTU Veterinærinstituttet. Formålet med minkundersøgelsen i denne afhandling var at bestemme, i hvor høj grad de danske minkfarme var MRSA-positive, og hvor på dyret man oftest kunne finde bakterien. Vi fandt, at MRSA CC398 var til at finde på en tredjedel af de testede farme, hvor bakterien som oftest fandtes i svælget eller på poterne af dyret. Dette udgør en human risiko for smitte, da minkavlere risikerer at blive bidt og revet, når de håndterer dyrene.


Overordnet set har denne ph.d. afhandling været med til at gøre det helt tydeligt, at det er vigtigt at få sænket niveauet af MRSA CC398 i den danske svineproduktion for at mindske spredningen fra svin til andre produktionsdyr. Ligeledes viser resultaterne, hvor vigtigt det er kontinuerligt at undersøge udviklingen af MRSA CC398 i reservoirer, hvor denne bakterie findes i lav grad samt nyligt opståede reservoirer. Dette er nødvendigt, hvis det skal være muligt bevidst at influere og kontrollere udviklingen samt spredningen af MRSA CC398 i Danmark, med det endelige mål at mindske fremkomsten af nye reservoirer som på sigt kan have konsekvenser for folkesundheden.
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>clonal complex</td>
</tr>
<tr>
<td>CHIPS</td>
<td>chemotaxis inhibitory protein of <em>S. aureus</em></td>
</tr>
<tr>
<td>CoNS</td>
<td>coagulase-negative staphylococci</td>
</tr>
<tr>
<td>IEC</td>
<td>immune evasion cluster</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence type</td>
</tr>
<tr>
<td>MPN</td>
<td>most probable number</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>methicillin-sensitive <em>S. aureus</em></td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NAM</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine leucocidin</td>
</tr>
<tr>
<td>SAB</td>
<td>bacteremia</td>
</tr>
<tr>
<td>SCCmec</td>
<td>staphylococcal cassette</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SCIN</td>
<td>staphylococcal complement</td>
</tr>
<tr>
<td>ST</td>
<td>sequence type</td>
</tr>
<tr>
<td>WGS</td>
<td>whole-genome sequencing</td>
</tr>
</tbody>
</table>

Important nomenclature

Throughout this thesis, the following terms are used as follows;

- **MRSA** = methicillin-resistant *S. aureus*, relates to all types (HA-, CA- and LA-MRSA) which are mecA positive
- **HA-MRSA** = hospital-associated MRSA, only relates to the types associated to hospital settings
- **CA-MRSA** = community-associated MRSA, only relates to types associated to the general community
- **LA-MRSA** = livestock-associated MRSA, used as a broad term which relates to all lineages associated to animals
- **MRSA CC398** = relates only to the specific lineage MRSA CC398
- **mecC-MRSA** = relates to MRSA carrying mecC and not mecA
List of manuscripts


Co-authorships related to, but not included in the thesis:


Conference contributions:


6. Hansen, Julie Elvekjær; Rhod Larsen, Anders; Skov, Robert Leo; Chriél, Mariann; Larsen, Gitte; Angen, Øystein; Larsen, Jesper; Corvera Kløve Lassen, Desireé; Pedersen, Karl / Widespread presence of MRSA CC398 in the Danish production of farmed mink (Neovison vison). The Danish Microbiological Society Annual Congress 2016. Copenhagen. Programme & Abstracts. p. 70-71 P54. Poster presentation.
1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as one of the most common multi-resistant bacteria found in both human and veterinary medicine (Dahms et al., 2014a). Livestock-associated MRSA (LA-MRSA) pose a risk of zoonotic infections in humans, most often in people with direct contact with contaminated livestock. Evidence has been presented demonstrating that LA-MRSA to some extent has the ability to colonize humans (McCarthy et al., 2012). In line with this, an increase in the occurrence of positive carriers has been observed in the general population in Denmark, in a manner which follows the increasing population of positive pig herds (DANMAP 2016). This development threatens the national low levels of MRSA in humans and the sustainability of the domestic ‘search-and-destroy’ control policies. This policy is based on infection control strategies at hospitals, which among other things depend on carriers being treated in isolation, where anyone entering the room wears face-nose mask and gloves. People transferred from foreign hospitals are housed in a single room and screened for MRSA and all carriers are treated with nasal mupirocin (Vandenbroucke-Grauls, 1996). Continuous increased dissemination into the general community and increased burden to the healthcare system are likely consequences if this development is not arrested.

The fast increase in the prevalence of positive pig herds in Denmark is well documented and it seems as we currently are not able to reverse this development (Miljø-og Fødevareministeriet, 2017). The actual within farm levels are not known or monitored. Establishment of quantitative studies of LA-MRSA in farms are needed to establish the level of LA-MRSA in different locations of farms, a knowledge which is important to gain before the transmission dynamics and effect of intervention strategies can be understood. However, multiple challenges arise regarding establishment of quantitative methods including that standardization is difficult. Nonetheless, robust methods are necessary in order to document a possible reduction in the presence of LA-MRSA within farms as a result of implementation of intervention strategies. Quantitative analysis of the farm level of LA-MRSA would also enable ranking of intervention possibilities in the primary production and environment.

*S. aureus* is an impressive bacterium which continuously amazes with its ability to adapt to new reservoirs and growth circumstances, a skill which has health consequences in relation to humans.
(Spoor et al., 2013). Dissemination of LA-MRSA from pigs into other animals has been observed repeatedly and emergence of new reservoirs increases the exposure to humans. The emergence and expansion of known and new clones, highlights the crucial importance of identifying potential new LA-MRSA reservoirs with possible relevance to human health (Fitzgerald, 2012).

1.1. Aims of the study

The current PhD study was initiated to elucidate the level of LA-MRSA within the Danish pig production and to validate quantification methods for LA-MRSA. Further, it was of interest to identify possible unknown animal reservoirs that may pose a risk to human health.

The following studies and aims were set up in order to obtain answers within the study purpose:

**Manuscript 1:**

The aim of study 1 was first of all to verify that LA-MRSA in pig farms can be quantified and to describe the level of MRSA CC398 in Danish pigs. Second, we aimed to examine whether air samples can be used as an alternative to nasal or skin samples as air samples are less laborious to acquire.

**Manuscript 2:**

This aim of study 2 was to investigate if Danish veal and dairy herds constitute a reservoir of MRSA CC398 and to possibly identify the source of introduction.

**Manuscript 3:**

The aim of study 3 was to determine the degree of LA-MRSA carriage in mink and to identify where the bacterium most often could be detected.

**Manuscript 4:**

The aim of study 4 was to investigate the emergence of MRSA CC398 in the mink production and the spread to humans working in the mink industry.
2. Background

2.1. Staphylococcus aureus

*S. aureus* is a Gram-positive opportunistic pathogenic bacterium, which is facultative anaerobic and a member of the phylum Firmicutes. It is known as the golden staph as it forms yellow colonies on blood agar. It forms grape-like clusters and persistently or intermediately colonizes 50% of the human population, where it primarily colonizes the nasal cavity (Williams, 1963). For this sub-population, it is recognized as a part of the commensal flora, and a given person is an asymptomatic carrier. Under the right conditions, *S. aureus* can cause infections ranging from superficial skin and soft tissue infections (SSTI), such as abscesses, to invasive infections as bacteremia (SAB), osteomyelitis and endocarditis (Gordon and Lowy, 2008). A fulminant progress of some of these conditions can be associated with high mortality, with an age-adjusted mortality at 2-10 deaths per 100,000 populations due to SAB infections recorded each year worldwide, a similar rate of deaths as for AIDS, tuberculosis, and viral hepatitis for comparison (van Hal et al., 2012). *S. aureus* can facilitate its access to tissue and bloodstream through virulence mechanisms, open wounds or it can utilize e.g. a catheter or surgical wound as entry size, which makes people with carrier status more likely to be infected than non-carriers (Lautenschlager et al. 1993; Wertheim et al. 2005).

2.2. Methicillin-resistant Staphylococcus aureus

In the 1940s, penicillin for medical use was introduced. Only two years after the introduction of penicillin, the first penicillin resistant *S. aureus* was seen within hospitals, followed by resistance observed in the general community. In 1960, 80% of all *S. aureus* strains were estimated to confer resistance towards penicillin (Deurenberg and Stobberingh, 2008). Penicillin is a group of antibiotics, which mode of action is to inhibit cell wall synthesis, and it is the drug of choice towards treating non-resistant staphylococci and streptococci. All penicillins are β-lactam antibiotics, a class of broad-spectrum antibiotics, with a beta-lactam ring in their molecular structures. They function by binding to the penicillin binding protein (PBP), a protein responsible for the cross-linking of the peptidoglycan in the bacterial cell wall synthesis, resulting in inhibition of the construction of new cell wall (Abraham...
and Chain, 1988). The primary acquisition of resistance is achieved through uptake of a plasmid encoding an penicillin hydrolysing enzyme named penicillinase, which cleaves the \( \beta \)-lactam ring leaving the penicillin molecule inactive (Madigan et al., 2009; Turlej et al., 2011).

In 1959, semi-synthetic penicillin named methicillin was introduced. This \( \beta \)-lactam antibiotic is penicillinase stable and therefore able to treat \textit{S. aureus} otherwise resistant to penicillin (Deurenberg and Stobberingh, 2008; Grundmann et al., 2006). Within two years after the introduction of methicillin, the first methicillin-resistant \textit{S. aureus} was observed in the hospitals (Jevons et al., 1963). Methicillin-resistant is caused by the gene \textit{mecA}, or its homologue \textit{mecC}, present on a mobile genetic element called staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}). Eleven different SCC\textit{mec} elements have been identified, which are classified into types and sup-types that differ in the structural organization and genetic content (Liu et al., 2016). The \textit{mec} gene encodes a penicillin binding protein (PBP) called 2a, which results in resistance towards all \( \beta \)-lactams. Four native PBP’s are present in \textit{S. aureus} which are essential in formation of the mesh-like layer, which constitute the peptidoglycan layer found in the cell wall, formed by the cross-linking of the two amino-sugars, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). The native PBP’s have a high affinity towards \( \beta \)-lactams, and upon treatment the biosynthesis of the peptidoglycan layer is disrupted when \( \beta \)-lactam bind to PBP, resulting in cell lysis. However, PBP2a has a low affinity for \( \beta \)-lactams and take over the function of the native PBP’s in \textit{mecA} positive strains, ensuring continuance of proper cell wall synthesis (Guignard et al., 2005; Ito et al., 2003). Since the discovery of hospital-associated MRSA (HA-MRSA), various clones with different genetic background and SCC\textit{mec} types, have successfully disseminated worldwide. However, the prevalence of MRSA in hospital settings largely differs between countries, with the lowest prevalence found in Northern European countries at \(<5\%\) (Deurenberg and Stobberingh, 2008; Tiemersma et al., 2004). The recorded prevalence in southern Europe is as high as 45\%, while reports of prevalence in eastern Asia > 70\%, USA, South America, Portugal and Malta > 50\%, Australia, China, Africa and most of Europe > 20\% (Stefani et al., 2012).

In the 1990s, another type of MRSA was found to flourish, called community-associated MRSA (CA-MRSA). This type is highly virulent, characterized by expressing the \textit{S. aureus} specific exotoxin named Panton-Valentine leucocidin (PVL) associated with SSTI. CA-MRSA has as well spread worldwide, first in the community, but later it has also been observed to thrive in the health care system
CA-MRSA and HA-MRSA are closely related assessed from the genotypes, however they carry different SCCmec elements and CA-MRSA tend to be less resistance towards other types of antibiotics than β-lactams. Over time, the distinction between CA-MRSA and HA-MRSA has become less obvious and now CA-MRSA largely contributes to the burden of MRSA in hospitals. As a result, the resistance expressed by CA-MRSA in hospital settings has accumulated making CA-MRSA an even bigger threat to the public health and increase the economic burden on the healthcare systems (Otter and French, 2011). This is a fine example of how one type of MRSA has been able to successfully disseminate from its original reservoir into a new one.

MRSA have evolved over time, first recognized as a problem associated to healthcare institutions, to a highly virulent and successful pathogen disseminating in the community. The most recent lineage of MRSA has progressed into livestock on a global level, asymptotically colonizing different animal productions. This jump was first recognized in the beginning of year 2000. An issue which receives a lot of attention, is to find out the risks that LA-MRSA pose to society, and to gain perspective on the consequences it could have in global health (Cuny et al., 2015b; Price et al., 2012).

2.3. Livestock-associated MRSA

The first case of MRSA in animals were described already in 1972 where MRSA was detected in milk from a cow with mastitis (Devriese et al., 1972). Since then MRSA has disseminated worldwide and currently many different animal types represent a reservoir of MRSA, which has led to the designation livestock-associated MRSA (LA-MRSA) (Cuny et al., 2010). Based on different typing methods, MRSA can be assigned to belong to a clonal complex (CC), sequence type (ST) or spa type. CC and ST is determined by multilocus sequence typing, based on sequencing of seven housekeeping genes, and spa type is determined by sequencing of the polymorphic region in the staphylococcal surface protein A (spa). Both typing methods will be described further in section 2.7.

Different lineages of LA-MRSA with animal origin have been found, some with less host specificity than others, however most lineages have been identified from multiple hosts. Numerous different MRSA lineages have been found in animals and are known to originate from either humans (HA-
MRSA and CA-MRSA) or animals (Cuny et al., 2015b). Only a few lineages, chosen to give a brief overview of host diversity, will be highlighted in the following.

MRSA CC398 is the most widely disseminated type of LA-MRSA to date (Cuny et al., 2015b), and will be presented in section 2.4 dedicated MRSA CC398. The LA-MRSA lineage found second most frequently is CC9 which is most prevalent in livestock of different animal species in Asia, primarily in pigs, however it has been found worldwide, but in lower prevalence compared to Asia (Chuang and Huang, 2015). CC1 has been found to have low host specificity and is originally known as a human MRSA being PVL positive and emerged in the US around 1990 (Deurenberg and Stobberingh, 2009; Grundmann et al., 2006). In animals, CC1 was observed in Hungary causing subclinical mastitis in dairy cattle and since 2010 it has been seen to increasingly cause mastitis in Italian dairy cattle, and has also been detected in bulk tank milk, goat milk, calves and small ruminants (Alba et al., 2015; Cortimiglia et al., 2015). CC1 has also been found in horses in Austria (Cuny et al., 2008) and in pigs from multiple European countries (Alba et al., 2015). ST 5 have been reported from the USA to be highly prevalent in the pig production (Frana et al., 2013; Molla et al., 2012). Interestingly, methicillin-sensitive S. aureus (MSSA) belonging to ST5 has previously been associated with poultry. In a study including isolates from 4 continents, a recent human-to-poultry host jump followed by adaption and pandemic spread was discovered (Lowder et al., 2009). CC8 is recognized as one of the major HA-MRSA lineages circulating worldwide. However, it has been associated with horses in Europe and Canada and chickens in Belgium (Cuny et al., 2015b). In Switzerland, the emergence of MSSA CC8 of a bovine-adapted genotype has been found, which has lost the ability of colonizing humans (Sakwinska et al., 2011).

mecC-MRSA belonging to CC130, the most prevalent lineage of mecC, appear to have a highly broad host tropism, nonetheless it seems often to be associated to ruminants. To date, it has only been found within Western Europe. It has been observed in livestock, companion animals, marine mammals and wildlife, including pigs, dairy cattle, sheep, goat, dogs, brown rats, rabbits, wild hedgehogs, seals and captive mara with more. (Angen et al., 2016; Espinosa-Gongora et al., 2015b; Monecke et al., 2013a; Paterson et al., 2012; Petersen et al., 2013).
2.4. Livestock-associated MRSA CC398

2.4.1. Emergence

In the beginning of year 2000, a lineage of LA-MRSA emerged in the pig production known as MRSA CC398. From hereon after, MRSA was recognized as a problem in animals and this lineage has since disseminated worldwide. In 2008, a European survey in 26 countries found MRSA CC398 to be present in the pig productions in 17/26 countries, establishing MRSA CC398 as a highly successful lineage within pigs. The highest rates of MRSA CC398 were associated with countries with higher density of pig farming (Authority, 2009). Since the discovery of MRSA CC398, it has been isolated from various sources including horses, cattle, turkeys and chickens, however pigs seem to be the primary reservoir (Fitzgerald, 2012). Pigs are most often healthy carriers of MRSA CC398 and infections are rarely observed (van Duijkeren et al., 2007; Verkade and Kluytmans, 2014). The spread within farms depend primarily on skin-to-skin contact, which is a highly effective route of transmission as conventionally farmed pigs are kept closely together (Broens et al., 2012a, 2012b). Environmental contamination, multiple types of livestock on the premises, farm management and herd size are thought to be involved in within farm transmission of MRSA CC398 as well (Crombé et al., 2013). Increased farm hygiene, however, seems to be associated to low prevalence of LA-MRSA, hence possibly decreases spread (Graveland et al. 2010). Spread of MRSA CC398 throughout the pig production chain is seen, and animal trading seems to be an important factor for introduction of MRSA CC398 in naive farms, with an 11-fold higher odds ratio becoming MRSA positive if the supplier of piglets is MRSA positive (Broens et al., 2011).

2.4.2. Characterization

For molecular typing of MRSA, pulsed-field gel electrophoresis (PFGE) was previously considered as the “gold standard” (Strandén et al., 2003). MRSA CC398 is however non-typeable with the enzyme of choice for S. aureus, smal, as MRSA CC398 carries restriction modification system which modifies cleavage of the smal restriction enzyme leading to protection of digestion (Bens et al., 2006). By combining MLST and spa typing the emerging LA-MRSA lineage was identified as CC398 with numerous different spa types, to34 and to11 being most prevalent (Price et al., 2012; Vanderhaegen et al., 2010). Two types of SCCmec cassettes are primarily found in CC398 being type IV(2B or 2B&5) and type V(5C2&5)c, with Vc found as most prevalent. SCCmec Vc (Figure 1) contains a gene
encoding cadmium-zinc resistance, \textit{czrC}, and carries a plasmid, pT181, bearing an additional tetracycline resistance gene, \textit{tetK}. Most MRSA CC398 also carry the \textit{tetM} tetracycline resistance gene on Tn196-like transposon, in the chromosome (Li et al., 2011; Price et al., 2012).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{schematic_structure.png}
\caption{Simplified schematic structure of SCC\textit{mec} type V(52C&5)c. The cassette contains the IS431-\textit{\Delta mecRI-mecA}-IS432 operon and is integrated into the open reading frame \textit{orfX} (the open reading frame of which SCC\textit{mec} elements occur). The regulatory genes (\textit{mecRI}) have been interrupted by the insertion of IS431 resulting in a de-repression of \textit{mecA}. It contains the cassette chromosome recombinase (\textit{ccrC}) gene complex type 5 allele 8 and allele 2 responsible for insertion and excision of the cassette along with a \textit{czrC} gene encoding resistance towards cadmium and zinc. The plasmid pT181 carry the additional tetracycline resistance gene \textit{tetK}. Figure adapted from (Li et al., 2011) and previously published in a master thesis by Julie Hansen (2014).
}
\end{figure}

The genes \textit{tetM} and \textit{tetK} confers resistance via two different resistance mechanism, where \textit{tetM} encodes a ribosomal protection protein and \textit{tetK} encodes an efflux pump (Chopra and Roberts, 2001). Additional resistance against macrolides, lincosamides, aminoglycosides, trimethoprim and to a lesser extent fluoroquinolones have been documented in MRSA CC398 (Vanderhaegen et al., 2010). The presence of a \textit{mecA} negative SCC upon excision of \textit{mecA} by homologue recombination have been observed, leaving MSSA CC398 upon loss of \textit{mecA} almost as resistant as MRSA CC398 (Vandendriessche et al., 2014).

\subsection{2.4.3. Evolution}

In 2012 a study, based on whole-genome sequencing (WGS), reported MRSA CC398 to originate from an human MSSA ancestral lineage (Price et al., 2012). The WGS based phylogeny suggest, that upon a host jump and switch in niche of MSSA CC398 from human to livestock, the lineage lost the human
associated bacteriophage φSa3 as a part of adaption to a non-human host and subsequently acquired the SCC\textit{mec} cassette and \textit{mecA} (Price et al., 2012). The acquisition of SCC\textit{mec} is thought to occur fairly often resulting in multiple types of SCC\textit{mec}, further supported by the fact that the CC398 lineage have been shown to lack the type I restriction modification system, enhancing its ability to take up foreign DNA (Price et al., 2012; Waldron and Lindsay, 2006). Coagulase-negative staphylococci (CoNS) has been suggested to be the original source of SCC\textit{mec} in the farming environment (Hanssen and Ericson Sollid, 2006). Yet, acquisition of staphylococcal toxins by CC398 seems to happen rarely as this lineage typically lack toxin genes (Ballhausen et al., 2017). Generally, most MRSA CC398 does not carry the virulence factor PVL and MRSA CC398 is therefore described as less virulent compared to CA-MRSA, where high virulence is associated with the expression of PVL. (Argudín et al., 2011; Fessler et al., 2010; Gómez-Sanz et al., 2010; Gu et al., 2015; Kadlec et al., 2009; Koyama et al., 2015; Monecke et al., 2013b; Vincze et al., 2014; Wendlandt et al., 2013a).

As aforementioned, the majority of MRSA CC398 carries two tetracycline resistance genes, \textit{tetM} and \textit{tetK}, together with the gene, \textit{czrC} that confers resistance towards zinc. It is therefore believed that the selective pressure of antimicrobials, with excessive use of both tetracyclins and zinc oxide, in the pig production has led to the evolving and spread of MRSA CC398 in exactly this animal niche (Broens et al., 2012b; Cavaco et al., 2011; Larsen et al., 2016a; Slifierz et al., 2015a, 2015b).

2.4.4. Colonization and host specificity

Generally, \textit{S. aureus} strains able to colonize humans, HA-MRSA, CA-MRSA or MSSA, carry human-specific immune evasion factors. These factors represent an innate immune evasion cluster (IEC), present on a β-hemolysin (\textit{hlb})-converting bacteriophage, called φSa3. The bacteriophage carries multiple immune evasion molecules, including staphylokinase (\textit{sak}) and enterotoxin A (\textit{sea}), and immune modulators, chemotaxis inhibitory protein of \textit{Staphylococcus aureus} (CHIPS) and staphylococcal complement inhibitor (SCIN), all involved in the innate immune response. Multiple variants of IEC have been discovered carrying different combinations of the genes (McCarthy et al., 2012; van Wamel et al., 2006).

Based on WGS analysis of \textit{S. aureus} CC398, the lineage can be separated in different clusters (Price et al., 2012; Stegger et al., 2013) resulting in a division of two groups being a human-clade and a pig-
clade (McCarthy et al., 2012), respectively. Isolates found in the pig-clade are characterized by lack of φSa3, a result of adaptation to the animal host in the evolution of MRSA CC398. Absence of the human immune modulators is associated with MRSA CC398 being a poor persistent colonizer of most humans (Cuny et al., 2015a; Jung et al., 2017; McCarthy et al., 2012). Studies of short term exposure (1-3h) of humans visiting pig farms have shown a high degree of contamination of visitors directly after farm visits, followed by a rapid decline in the hours just after the visits and with 94-100% found MRSA negative between 24-48h post visits (Angen et al., 2017; Frana et al., 2013). In cases of long term exposure as seen with pig farmers, the dynamic might be different. A German study found the period of carriage to be prolonged in a majority of pig farmers (59%), who were found MRSA CC398 positive after their holiday (~10 days) (Köck et al., 2012). Several examples of re-acquisition of φSa3 resulting in human colonization have been reported, demonstrating that the presence of IEC facilitates animal-to-human jump (McCarthy et al., 2012; Price et al., 2012; Stegger et al., 2013), however these events seem to be rare at this point. In the Netherlands, a study recently reported on emergence of a possible sub-clade of LA-MRSA which resembles non-LA-MRSA regarding transmissibility and pathogenicity, able to spread in humans in areas where the density of farms are relatively low and where LA-MRSA is not the predominant MRSA variant (Bosch et al., 2016). Interestingly, this sub-clade exhibited low prevalence of φSa3 indicating the human associated phage, at least in this sub-lineage, to play a limited role in adaptation of LA-MRSA to humans as host (Bosch et al., 2016). Evidence of livestock as a reservoir of human pathogenic S. aureus has previously been provided, showing a S. aureus lineage of bovine origin, CC97, which became a human pandemic clone of CA-MRSA upon acquisition of φSa3 (Spoor et al., 2013). It is therefore not unlikely, that in time, an increase in human spread and transmission of MRSA CC398 may be observed, as a result of re-acquisition of φSa3 and re-introduction to its original human host.

2.5. Livestock-associated MRSA in humans

The global consequences of the emergence of LA-MRSA are not fully understood and the impact of the widespread reservoir of LA-MRSA for humans is currently under investigation in many countries. In most countries, the burden of MRSA CC398 in relation to the overall number of MRSA infections in humans, are at this point limited. However, in countries with a national low prevalence of human
MRSA cases, such as Denmark and The Netherlands, the burden of MRSA CC398 is found to be more profound (Guardabassi et al., 2013). The zoonotic potential of LA-MRSA have been established in epidemiological studies (Köck et al., 2013; Lewis et al., 2008), and transmission of LA-MRSA to people in low level MRSA countries would represent a threat to the public health and the sustainability of the national ‘search-and-destroy’ control policies of the respective countries (Guardabassi et al., 2013; Köck et al., 2010).

2.5.1. Infections

In Europe, especially in countries with intensive livestock production, MRSA CC398 is recognized as a frequent cause of disease in humans (Becker et al., 2017; van Cleef et al., 2011). Outside of Europe, infections caused by LA-MRSA have as well been reported from Japan, China, Tunisia, Canada and the USA (Barguellil et al., 2015; Casey et al., 2014; Golding et al., 2010; Koyama et al., 2015; Stegger et al., 2010). Infections caused by LA-MRSA are various as seen from MSSA, including several types of more or less severe SSTI, pneumonia, joint infections, bacteremia et cetera (Becker et al., 2017).

*mecC*-MRSA CC130 has also been found to cause infections in humans, mostly SSTI, however more severe infections such as bone infections (Barraud et al., 2013), nosocomial pneumonia (Cuny et al., 2011) and bacteremia (Garcia-Garrote et al., 2014) have also been reported. Infections caused by CC130 seem to occur more rarely than infections from different types of *mecA*-MRSA (Cuny et al., 2011). Some countries have implemented increased screening procedures and awareness of risk groups upon hospital admission, which has caused the number of new LA-MRSA cases to increase. However the total number of infections in the respective countries have decreased simultaneously (Köck et al. 2013; DANMAP 2014; Larsen et al. 2015). However, if LA-MRSA is introduced into hospitals despite the selective screening procedure, nosocomial spread in immunocompromised patients can increase the number of infections within hospital settings (Wulf et al., 2008). Luckily, studies have shown MRSA CC398 to be 4 to 6-fold less transmissible within hospital settings compared to HA-MRSA (Bootsma et al., 2011; Wassenberg et al., 2011), however as pointed out by Graveland et al., (2011), these results should be used with caution as data on potential risk factors and patient characteristic are missing in the calculations.
2.5.2. Transmission

Transmission of MRSA CC398 between humans and livestock, e.g. pigs, cattle, horses and poultry have been reported in numerous cases (Dahms et al., 2014b; Geenen et al., 2013; Graveland et al., 2011a; Islam et al., 2017; Lewis et al., 2008; van Loo et al., 2007; Witte et al., 2007). LA-MRSA is primarily transmitted to humans with contact to LA-MRSA positive animals, making farms workers and veterinarians risk groups of colonization. Household members, with indirect animal contact, at farms are less frequently colonized compared to farmers (Cuny et al., 2009; Smith et al., 2013; van Duijkeren et al., 2010). Studies from both veal and pig farming have shown, direct animal contact and the intensity and duration of contact to be important risk factors, which strongly increase the prevalence of carriage (Graveland et al., 2011b; van den Broek et al., 2009).

A German study identified direct animal contact as the most important risk factors for MRSA CC398 acquisition, however they saw a considerable proportion of their study population who seemed to acquire MRSA CC398 through other pathways (Deiters et al., 2015). In countries with extensive pig production, such as Denmark, The Netherlands and Germany, a steady proportion of cases with MRSA CC398 (11-15% of all reported cases and 37% of all reported infections) is observed in people without exposure to livestock (Lekkerkerk et al. 2012; DANMAP 2015; Larsen et al. 2015). In line with this, epidemiological studies have found regional density of livestock (pigs and veal calves) and proximity to farms, to pose a risk factor for carriage of MRSA CC398 in nasal cavities of people with and without contact to livestock (Feingold et al., 2012; van Cleef et al., 2011). Likewise, human cases of MRSA CC398 in some areas have been shown to correspond to the density of pig farming while the density of other types of MRSA corresponds to the density of human population (van Loo et al., 2007). Results from these studies suggest the presence of MRSA CC398 in the general community as a consequence of spillover from the surrounding farms, and that an expansion of the livestock reservoir of LA-MRSA, while MRSA-naive farms become infected, is likely to increase the extend of spillover. Transmission outside of farms could be spread of LA-MRSA via air from farm ventilation systems (Gibbs et al., 2006; Schulz et al., 2012), through fertilization of agricultural fields (Casey et al., 2013) or by human-to-human transmission as it is seen for other S. aureus strains (David and Daum, 2010), making livestock workers a possible important source of delivery of MRSA CC398 to otherwise unexposed people.
Transmission can also occur through contaminated meat and slaughterhouse workers have been found to have increased prevalence in carriage of LA-MRSA (Gilbert et al., 2012; Mulders et al., 2010; van Cleef et al., 2010). MRSA is found frequently in meat products worldwide (Figure 2) and thereby it has the potential of being an important route of transmission to the general population (Kluytmans, 2010; Wendlandt et al., 2013b), however it would be lack of kitchen hygiene which pose the actual risk of becoming infected as LA-MRSA rarely carry the heat stable staphylococcal enterotoxins (Ballhausen et al., 2017).

![Image](image.png)

**Figure 2** | Overview of the various types of methicillin-resistant *S. aureus* detected in food originating from different countries. From Wendlandt et al., (2013b).

One example is the result of a survey conducted by the Dutch Food and Safety Authority analyzing the presence of MRSA in around 1,300 retail meat products where a prevalence of 11% was found. This included meat from turkey (31% positive), chicken (27% positive), veal (17% positive) and pig (10% positive) (Verkade and Kluytmans, 2014). A study by de Boer et al., (2009) also showed a high prevalence of MRSA, however in low numbers, in meat from different animal sources. Moreover,
despite the extensive presence of LA-MRSA in meat, LA-MRSA in unexposed people in urban areas is rare. Therefore, food-borne transmission of LA-MRSA is thought to be of minor importance and not to play a role in the epidemiology of LA-MRSA in the general community (Larsen et al., 2015; van Loo et al., 2007). However, one study has shown carriage of MRSA to be associated with regular consumption of poultry in a case-control study (van Rijen et al., 2013). Also, another study showed evidence of human adaption and food-borne transmission of a CC9/CC398 hybrid strain originating from poultry (Larsen et al., 2016b). Yet, at this point, there is not sufficient data to change the general accepted role of food-borne transmission in human carriage of LA-MRSA as negligible, concluded by Larsen et al., (2016b).

The ability of S. aureus to adapt to a broad range of different species including humans and various animals, emphasize the necessity of continued surveillance of LA-MRSA to detect changes of evolutionary and epidemiological importance, e.g. related to emergence of new reservoirs, to consequently enable quick implementation of interventions to protect human health.

2.6. Dissemination into new animal reservoirs

The primary reservoir of MRSA CC398 is pigs. As aforementioned the lineage has within a short time period successfully disseminated to other livestock reservoirs. There are multiple possible routes of introduction into naive farms. Human introduction into LA-MRSA negative farms have been shown in Norway upon eradication and sanitation of farms (Grøntvedt et al., 2016), which illustrates humans, such as veterinarians, consultants or employees, as a possible dissemination route into new animal reservoirs. LA-MRSA has been found in rodents on pig, poultry, veal and goat farms, and might act as a transmission route, as rodents potentially play a role in transmission a zoonotic bacteria on livestock farms (Meerburg et al., 2006). One study found positive rats on a mixed pig and veal farm where transmission between species via rats would be possible (van de Giessen et al., 2009). Further, transmission via environmental contaminations could occur on farms with multiple types of livestock on the premises as dust and air in LA-MRSA positive productions are contaminated with viable LA-MRSA. Airborne LA-MRSA is also a possible route of transmission to reach neighbor farms in close proximity rural areas (Authority 2009; Friese et al. 2012; Friese et al. 2013; Bos et al. 2014).
Houseflies, which flight distance ranges between 5 and 7 km, could possibly also facilitate transmission as MRSA CC398 was found to survive in contaminated flies for up to 3 days upon removal to an MRSA free environment (unpublished data). A German study supports this suggestion, as a LA-MRSA positive fly was found in the city center of Münster, as part of a screening of flies from urban and rural areas near Münster (Schaumburg et al., 2016). The suggested transmission routes are all likely to depend on LA-MRSA being heavily present in one reservoir, such as pigs, to result in spillover into naive livestock productions.

2.7. Typing methods and tracking of MRSA dissemination

Different molecular typing methods have proved able to identify and classify MRSA species. Two methods often used are multilocus sequence typing (MLST) and spa typing.

2.7.1. MLST

MLST is based on sequence analysis of seven housekeeping genes and is a highly discriminatory typing method. Alleles in each housekeeping gene define an unique allelic profile (genotype) which is assigned a sequence type (ST). Sequence types which are highly similar, share alleles at ≥5 loci, can be placed together and considered to belong to the same clonal complex (CC) (Enright et al., 2000). The housekeeping genes have been shown to be highly stable and are therefore less suitable for tracking of outbreaks or short-term epidemiological studies and more suitable for typing and long-term epidemiological investigations (Spratt, 1999).

2.7.2. spa typing

spa typing is a sequence based typing method targeting the variable polymorphic region (known as region x) of the gene encoding staphylococcal surface protein A (spa). This region is composed of a number of repeats varying from 1-27, showing more or less sequence variation, and differs in size (21 bp to 30 bp). The order of the repeats define the spa type of an isolate (Hallin et al., 2009). Analysis has shown a limited number of spa types to be associated to a given MLST type and spa typing is usually cheaper, faster and more discriminatory than MLST, able to assign different spa types based on a single single-nucleotide polymorphism (SNP) (Monecke et al., 2011; Wendlandt et al., 2013b). spa typing has been validated for outbreak investigations (Shopsin et al., 1999).
2.7.3. Whole-genome sequencing

The process of whole-genome sequencing determines the complete DNA sequence of the genome of an organism. Based on the detected SNPs) and their distribution within the genomic material, it is possible to determine the relationship between isolates. For instance, insight into bacterial evolution, clone emergence and expansion, and the molecular basis of niche adaptation can be obtained when comparing sequences from WGS. This is of great value in research, and studies using WGS to track outbreaks in near real-time or retrospectively have been published (Bal et al., 2016; Ng and Kirkness, 2010). Multiple studies based on WGS have provided valuable information regarding host switching and emergence of MRSA lineages. This includes the previously described evolution of MRSA CC398 (Price et al., 2012), host jump and adaption of ST5 (Lowder et al., 2009) and CC97 (Spoor et al., 2013). These studies highlight the ability of S. aureus to cross species barriers and capacity to adapt to new niches, and the critical importance of detecting and identifying potential reservoirs of emerging strains.

The procedure

Whole-genome sequencing can best be described in steps and the process, described in short, starts with purification of genomic DNA followed by tagmentation, where the DNA is fragmentized and tagged with adaptor sequences essential in the following PCR step. Next follows the amplification step, resulting in clusters of single stranded DNA, where index adaptors and common adapters, are added to each end of the fragment, enabling sequencing from both ends of the DNA. Common adaptors are used for adherence to a flow cell, while index adapters contain the sequencing primer site required for sequencing and enable the identification of a specific sample afterwards. Last, each fragment is sequenced utilizing fluorescent labelled nucleotides which bind, one by one, to the bases of the fragment (Loman et al., 2012).

2.8. Livestock-associated MRSA in a selection of Danish production animals

In this section an overview of the Danish situation regarding LA-MRSA will be provided. A review of the Danish situation is relevant, as the manuscripts included in this thesis all revolves around Danish production animals. A review of the challenges regarding quantification of LA-MRSA is included as a
subsection, however, these challenges are not unique for the Danish situation, but included in the following, as it is of relevance to the choice of method applied in the study described manuscript 1.

2.8.1. Conventional pigs

MRSA CC398 was first reported in Denmark in 2008 where 3% of the screened farms were found positive (Authority, 2009) and already in 2014, the prevalence was found to be 68% (DANMAP 2014). In 2016, a screening conducted by the Danish Veterinary and Food Administration found the prevalence of positive pig herds to have increased to 88% which means that the pig production at this point constitutes a major reservoir of LA-MRSA in Denmark (Figure 3) (DANMAP 2016).

![Figure 3](image)

**Figure 3** | Number of MRSA CC398 infectious cases in people with and without livestock contact, combined with the percentage of positive pig herds (DANMAP 2016).

Some of the farms found negative in the screening in 2014, was included in the screening in 2016, and 62% of production herds and 100% of breeding herds which were found LA-MRSA negative in 2014, where found LA-MRSA positive in 2016. No new pigs had been introduced to the new positive breeding herds since last screening. Furthermore, all farms found LA-MRSA positive in 2014, were
still positive in 2016 indication no spontaneous elimination of LA-MRSA on conventional pig farms (DANMAP 2016). This rapid increase suggest that LA-MRSA thrives in Danish pigs and that transmission into naive farms indeed occurs despite the increased awareness and focus on LA-MRSA in production animals which has developed over the same time span.

MRSA CC398 is the most prevalent lineage in Denmark with t034 being dominant followed by t011, and CC1 has been found on a limited number of farms (Sieber et al. unpublished data). A single farm has been identified as positive for mecC-MRSA belonging to CC130 (Angen et al., 2016).

Zinc is used, in low doses, as a feed additive to prevent post-weaning diarrhea and in higher doses as a treatment. Tetracycline is the most frequently used antibiotic in the pig production with a consumption of more than 22 tons a year (DANMAP 2016). As aforementioned, within herd usage of tetracyclines and zinc impose an important selective pressure of MRSA CC398 (Larsen et al., 2016a; Moodley et al., 2011).

2.8.1.1. Quantification of MRSA CC398

No one doubts the high prevalence seen in positive pig herds, however the actual load of LA-MRSA inside farms has not been thoroughly investigated yet. Multiple challenges arise regarding establishment of quantitative methods targeting LA-MRSA which complicates such investigations. First of all, it is difficult to obtain standardized sample material originating from pigs. When taking samples from live pigs, the animals move away. As a result, the total amount of sample material on each swab or wipe will be different and this variation will influence the end result, no matter which approach for quantification is applied. In contrast, environmental samples are easier to standardize. One can obtain a specific volume of air, swipe a specific surface area, and collect settled dust over a specific time period.

An often utilized method for quantification of bacteria is qPCR, where the target DNA is monitored during PCR in real time. This method can be used for precise quantitative measurements of the target DNA by detection of fluorescent reporter molecules. Yet, a number of problems occur regarding quantification of LA-MRSA by qPCR, one being the fact that it is not possible to quantify MRSA with a single PCR target. It is possible to monitor multiple targets, in a multiplex reaction, by applying different fluorophores, however this does not solve the problem regarding LA-MRSA as samples
retrieved from a farm environment is heavily loaded with multiple bacterial species. The following subsection elaborates on these problems related to qPCR on LA-MRSA.

MRSA CC398 found in Danish pigs, primarily carry the SCCmec cassette type V(5C2&5)c which consists of 43,381 bp (Li et al., 2011). An obvious target for qPCR would be mecA, however this gene can also be found in coagulase-negative staphylococci present in pig farms such as *S. haemolyticus*. A qPCR could be developed to target both the mecA gene and the *S. aureus* core genome outside of the SCCmec, but this would not be possible in practice, as the size of the PCR product would be too big to amplify. Another target could be the open reading frame of which SCCmec cassette occur, Orf-X, thereby targeting *S. aureus* with the SCCmec cassette (Gilbert et al., 2012), which would represent *S. aureus* carrying the cassette, however mecA empty cassettes have been observed which will be included in the quantification leading to over-estimation (Vandendriessche et al., 2014). The level of isolates harboring remnant DNA of SCCmec V(5C2&5)c element, which lack the mec gene complex within pig productions is not known, and the influence on the quantification posed by the false positive isolates are therefore difficult to assess. Further, this qPCR would have to be adapted to the different cassettes present in pigs, however, in Denmark, this would not be a big issues as >95% of CC398 carry a single type of cassette (Sieber et al., unpublished data). One study quantifies MRSA CC398 on the basis of four targets, femA/nuc (*S. aureus* specific), mecA and C01 (a target specific for *S. aureus* CC398) (van Cleef et al., 2014). The concentration of MRSA CC398 is estimated based on detection of the mentioned targets in relation to on another. However, the amount of MRSA CC398 may be overestimated due to CoNS carrying the mecA gene or as qPCR also detects non-viable bacteria (Figure 4). None the less, currently it is the best qPCR method available for quantification of MRSA CC398 from a farm environment.

![Figure 4](image-url) | Two examples of possible overestimations of MRSA CC398 by the qPCR described by van Cleef et al., (2014).
A Danish study tried to quantify the level of MRSA CC398 within pig farms (Espinosa-Gongora et al., 2015a). This study utilizes a culture based method known as most-probable-number (MPN), where the amount of bacteria is estimated on the basis of multiple sample dilutions, and detection of growth/no growth on plates or in liquid. MPN is estimated from the highest dilution showing growth and yields a semi-quantitative result presented as confidence intervals. To utilize this approach with regard to intervention strategies against MRSA CC398, a reduction in load from one interval to another would have to occur, in order to be reflected in the MPN result, whereas a little reduction in load might be overlooked. One other culture based method is direct plating of sample material followed by colony count, which is an easily applicable and low cost method for quantification. From this method it is possible to quantify the total amount of viable MRSA CC398, if selective plates are used, and detect smaller changes in load compared to MPN. Based on this brief review of possible methods for quantification of MRSA CC398 within Danish pig farms, direct plating would be the method, which introduces fewest challenges and could be implemented in practice fast and at a low cast.

An attempt to describe the level of MRSA CC398 in Danish pigs based on quantification by direct plating of different animal samples combined with colony count of air samples, has been conducted as a part of the present PhD project, and results hereof are represented in manuscript 1.

### 2.8.2. Free-range and organic pigs

In 2015, the Danish Veterinary and Food Administration conducted a survey of organic pig herds and found only 6% to be LA-MRSA positive. A research study, testing the population dynamics of LA-MRSA in free-range pigs, with sample population of five organic and one conventional free range farm, was carried out in 2016. The farms were tested multiple times to examine the population dynamics of LA-MRSA after introduction of positive conventional breeding pigs. Interestingly, it seemed like a spontaneous elimination of LA-MRSA took place on the free-range farms, as four of the five farms, which received MRSA positive pigs from conventional breeding farms, were tested MRSA negative after three month (DANMAP 2016). This is a completely different dynamic than observed in conventional pig productions.
2.8.3. Horses
A research study published in 2017, investigated whether horses constitutes a reservoir of LA-MRSA in Denmark (Islam et al., 2017). They collected samples from 401 horses originating from 74 farms and found a prevalence of positive farms at 9% (7/74) and positive horses at 4% (17/401), with the recently identified horse-adapted sub-lineage, CC398 t011, being dominant. Interestingly, four isolates of CC398 t034 were found to belong to the pig-adapted lineages seen in Denmark (Sieber et al., unpublished data) which indicate a spillover from the pig production into horses. The low number of positive samples could indicate contamination rather than actual colonization. Three samples was identified as mecC-MRSA CC130 (Islam et al., 2017).

2.8.4. Dairy cattle and veal calves
Very little is known regarding LA-MRSA in Danish cattle. LA-MRSA, belonging to CC398 and CC1, was detected in a limited number of bulk tank milk (BTM) samples in 2012 (2%, 4/219) (DANMAP 2012). In 2015, a screening of veal calf farms was conducted where 10% (5/50) farms was found positive for LA-MRSA (DANMAP 2015). These observations suggest dairy cattle and veal calves to be a possible reservoir of LA-MRSA in Denmark.

Additional screenings of both veal calves and dairy herds have been conducted as a part of the present PhD project, and results hereof are represented in manuscript 2.

2.8.5. Mink (Neovison vison)
Since 2009, people with contact to mink, have been found positive for carriage of LA-MRSA, which is an interesting observation as there is no obvious reason for why mink should constitute a reservoir of LA-MRSA. In 2013, MRSA was unexpectedly detected in a wound of a diseased mink during an autopsy at the National veterinary institute. Denmark represents the largest mink production worldwide with a production of 31% of the total world production of pelted skin, and around 6,000 people are currently employed in the Danish mink industry¹,². If the mink industry represents a reservoir, these people are potentially exposed to LA-MRSA and at risk of becoming carriers.

² Danske minkavlere. Available online at: http://www.danskeminkavlere.dk/fakta-om-branchen/
As a part of the present PhD project, investigations of mink as a possible newly emerged reservoir of LA-MRSA was carried out, results hereof are represented in manuscript 3 and 4.
3. Manuscripts
Quantitative assessment of MRSA CC398 in the Danish pig production

Hansen JE, Sørensen AIV, Pedersen K, Angen Ø, Larsen AR and Strube ML

Manuscript in preparation.

Introduction to the study

The prevalence of MRSA CC398 in Danish pigs is high, however, there is very little information available on the actual levels of MRSA CC398 within farms. To gain knowledge regarding within farm infection levels is of great importance as it would enable assessment of different intervention strategies. MRSA CC398 can be transmitted out of pig-farms via spillover of MRSA CC398 into the general human community and via expansion of MRSA CC398 into new animal reservoirs. Such transmission of MRSA CC398 might pose a threat to human healthcare. Many aspects contribute to the risk of MRSA-transmission. These aspects include e.g. the exit hygiene level for humans leaving a pig farm and the inherent properties of the MRSA CC398 including its capability to colonize humans. However, the prevalence of MRSA CC398 positive pig-farms and the within-farm level of MRSA CC398 might also be important aspects affecting the risk of spillover to both humans and animal reservoirs, respectively. Thus, a reduction in the within-farm levels might decrease the risk of transmission of MRSA to humans.

The following study was initiated to describe the level of MRSA CC398 in Danish pigs and to verify that MRSA CC398 in pig farms can be quantified by plate counting of both animal samples and air samples.
Quantitative assessment of LA-MRSA CC398 in Danish pigs

Julie Elvekjær Hansen\textsuperscript{a}, Anna Irene Vedel Sørensen\textsuperscript{a}, Karl Pedersen\textsuperscript{a}, Øystein Angen \textsuperscript{b}, Anders Rhod Larsen\textsuperscript{b}, and Mikael Lenz Strube \textsuperscript{a,*}

\textsuperscript{a}National Veterinary Institute, Technical University of Denmark, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark

\textsuperscript{b}Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark

Intended for: Applied microbiology

*Corresponding author:

Mikael Lenz Strube

Technical University of Denmark

National Veterinary Institute

2800 Kgs. Lyngby

Denmark

E-mail: mlst@vet.dtu.dk
ABSTRACT

Livestock-associated methicillin-resistant *Staphylococcus aureus* have rapidly spread worldwide and is highly prevalent in different animal productions with pigs constituting a major reservoir. Once established in a pig herd, the bacterium seems to persist, and elimination is both challenging and costly. An alternative to eradication of LA-MRSA may therefore be a lowering of the level within farms and limiting the spread into the community. In order to estimate the effect of possible intervention strategies, quantification of MRSA within sections is essential in order to study the magnitude of association between the level of LA-MRSA and the risk of human exposure. The aims of the study were to describe the general level of MRSA in Danish pigs, and to investigate if air samples can be used as an alternative to nasal or skin samples, as air samples are less laborious to acquire and would be more cost efficient compared to animal samples. A total of 1,120 nasal and skin samples were obtained from animals at three Danish farms and quantified by direct plating on MRSA selective agar plates. In addition, 40 air samples were collected with a Sartorius MD8 Air Airport. All sample types found MRSA loads to be highest in weaning and farrowing sections, and lower in sections with gilts and gestating sows. We found the air load of a section to be a better proxy for determining the level of MRSA in the nose of animals than on the skin, however not sufficient to recommend only taking air samples to monitor the level of MRSA. Air sampling is nevertheless a relatively easy and inexpensive method to assess a low or high MRSA status of sections and farms.
INTRODUCTION

A high prevalence of livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), especially in the pig production has been reported from many countries (Cuny et al. 2015).

Epidemiological studies have established the zoonotic potential of LA-MRSA and substantiated the transmission from pigs to humans (Lewis et al. 2008; Köck et al. 2013). Different types of MRSA, hospital-acquired, community-acquired and LA-MRSA, with different genetic backgrounds have disseminated worldwide. However, the overall level of MRSA within the society differs between countries, but some have generally low national levels of MRSA in humans, including the Netherlands and Scandinavia (Stefani et al. 2012). In these countries, an increased spread of LA-MRSA from the farm environment into the society represents a public health hazard and increases the economic burden to the healthcare systems by challenging the national ‘search-and-destroy’ control policies (Köck et al. 2010; Guardabassi et al. 2013).

In a study by Porphyre et al. (2012), simulations showed that it would be impossible to eliminate LA-MRSA from the community once the infection is established in pigs. Therefore, control measures should be focused on reducing the level of MRSA within positive farms in order to lower transfer from pigs to humans (Porphyre et al. 2012). The time spent in the barns and the intensity of contact to the animals are acknowledged as risk factors for transmission to humans (Cuny et al. 2009; Graveland et al. 2011). Furthermore, Angen et al. (2017) showed a quantitative correlation between LA-MRSA in barn air and contamination of human volunteers (Angen et al. 2017). There is however a lack of knowledge regarding the actual LA-MRSA loads on MRSA-positive farms as most studies use methods that do not allow quantitative assessment, but merely provides information on the presence or absence. A quantitative assessment of LA-MRSA levels in swine herds would facilitate investigation of the magnitude of association between the level of LA-MRSA and the risk of human exposure, and also
help to estimate the effect of intervention strategies. Furthermore, it is not known where on the pig it would be most relevant to sample in order to get a representative measurement of the load of LA-MRSA or whether environmental samples could substitute animal samples. Most prevalence studies use nasal swab samples, however, it has been suggested that skin swabs might be more sensitive (Agersø et al. 2013). An elaboration of the correlation between different samples types with quantitative data could indicate the most representative and cost efficient sampling types for assessment of LA-MRSA levels within farms.

The aim of the study was to evaluate the feasibility of quantitatively and reproducibly measure LA-MRSA in Danish pigs. Further, we investigated whether air samples can be used as an alternative to nasal or skin samples, as air samples are less laborious to acquire, which included an assessment of how well measurements of either the nasal and skin loads correlated to air loads.

MATERIALS AND METHODS

Sample collection and processing

Samples were collected from three different Danish pig farms, with production holdings of 1000-3000 animals on site. From farm 2 (animal sample, n=370), samples were obtained from all units including; gestation, farrowing, weaning and gilt. From farm 1 (animal samples, n=30) and farm 3 (animal samples, n=720), only samples from the weaning unit was obtained. From farm 2 and 3, samples were obtained from more than one section within each unit. The farms were visited between one and six times. The chosen farms were known MRSA positive farms and were selected by convenience and their willingness to participate. At each visit, nasal and skin swab (ESwab, Copan Diagnostics Inc., Murrieta, CA, USA) samples were collected from the animals while two air samples were obtained with a Sartorius MD8 Airport (Sartorius AG, Göttingen, Germany). From farrowing sections, samples
were obtained from both sows and piglets. A total of 40 air samples and 435 paired nasal (n=435) and skin (n=435) samples were obtained. In addition, a total of 250 individual nasal and skin samples were collected.

The nasal samples were obtained by swabbing with a rotary motion ~1 cm inside each of the nares three times with a single dry flocked swab. The samples referred to as skin samples were obtained by three gliding motions behind the pinna with a moistened ESwab. The samples were immersed in 1 ml transport media supplied with the ESwab immediately after sampling and transported to the laboratory where they were stored at 5 °C until analysis, and kept at 5°C in case of need for re-analysis. The samples were processed within 24 h after sampling. The samples were quantified directly by plating 100 µl transport medium undiluted and in 10⁻¹ dilution (in phosphate buffered saline) onto a selective MRSA plate (Brilliance MRSA2 agar, Oxoid, Basingstoke, UK) followed by incubation at 37 °C for 18–24 h. Colonies were counted and CFU/swab were calculated for each sample. In case of overgrowth, an additional dilution was performed within 48 h of sampling.

From each section, two air samples were collected 1.50 m above the floor while slowly walking the entire aisle of the section. The air sample was vacuumed onto a gelatin filter with a collection time of 5 min and an air flow of 50 L/min (a total of 250 L). Immediately after sampling the filter was placed onto a MRSA selective plate and after arrival at the laboratory, the plates were incubated at 37 °C for 18–24 h, after which colonies were counted and concentrations per m³ air were calculated. From all sample types, one presumptive MRSA colony from each sample was sub-cultured on agar plates (Oxoid, Basingstoke, UK) containing 5% calf blood for further verification. Isolates were identified as MRSA by PCR detection of the mecA and nuc genes (Maes et al. 2002).

If a sample was found MRSA negative by direct plating, 200 µl of sample material was enriched in Mueller Hinton broth supplemented with 6.5% NaCl and incubated for 18-24 h at 37°C. A loop full (10
µl) enriched broth was streaked onto MRSA2 plates and determined as MRSA-positive/negative after 18-24 h of incubation at 37 °C. In the data analysis, load in the samples that were only found positive after enrichment (nasal=48/610; skin=25/510) was set to 5 CFU/swab, which corresponds to half the detection limit of the quantitative test, whereas the load in samples where MRSA was undetectable after enrichment (nasal=11/610; skin=3/510) was set to 1 CFU/swab (corresponding to 0 upon log-transformation).

*Comparing nasal and skin swab loads*

To further investigate the relationship between nasal and skin loads, a method assessment of the two different sampling sites was performed. Three pigs from a weaning section were sampled with ESswabs in biological triplicates from behind the right ear, left ear, right and left nostril giving a total of 12 samples per pig. The samples were processed as described above, except that each sample was handled in technical triplicates.

*Statistical analysis*

All analysis were conducted in R version 3.2.2 ("Fire safety") (R Core Team, 2015). All analyses were therefore performed with log10-transformed data and analyzed with methods assuming normality (verified by inspecting residuals post-analysis). For some samples no quantitative results were available due to no growth by direct plating, however some of these samples were subsequently found positive upon enrichment.

*Comparing nasal and skin swab loads*

For the method assessment, data was analyzed separately for nasal and skin swabs, to see which sample type exhibited the lowest residual variance, e.g. in order to assess which sample type had more random
variation. The two datasets consisted of CFU counts based on the $10^{-1}$ dilutions when possible, however in cases of no growth on $10^{-1}$, CFU counts calculated from the undiluted sample was used. The data was fitted to a random effects model, specified using "lmerTest" (Kuznetsova et al. 2015) built on “lme4” (Bates et al. 2014). Fulfillment of the assumptions about normality and variance homogeneity for the residuals and normality of the random effects, were assessed using qq-plots and plots of residuals vs. predicted values. The proportions of variance between the samples that could be attributed to the different factors were then estimated from the following random effects model applied on each of the two separate datasets:

$$Y_{ij} = \mu + a_i + b_j + \varepsilon_{ij},$$

where $Y$ is logCFU on the $i$th swab, $\mu$ is the overall mean of all measurements for the given type of swab, $a_i$ is the random effect of the $i$th pig, $b_j$ is the random effect of the $j$th biological replicate, $\varepsilon_{ij}$ is the residual error term, and $a_j \sim N(0, \sigma^2_{\text{pig}})$, $b_j \sim N(0, \sigma^2_{\text{bio_rep}})$ and $\varepsilon_{ij} \sim N(0, \sigma^2)$.

**Differences in load across sections**

ANOVA was used to test for differences in log-mean CFU between the samples obtained from gestating sows, lactating sows, gilts, and weaning pigs in the farm with all units represented (farm 1). This was done individually for each of the three sample types (nasal, skin and air) and was followed up with Tukeys post hoc test if significant. Data was plotted using the package “Beeswarm” (Eklund, 2015).

**Association between load in air and on the animals**

Pearson correlations were calculated for the association between the load in air samples and the mean load in skin and nose samples for the given air sample. If significant, these were followed up with linear regression with zero intercept.
RESULTS

A comparison of two different sample types, nasal and skin swabs, was performed to assess the difference in residual variance between the sample types and to clarify what sample type was most reproducible. The analysis showed that the nasal swabs had a higher proportion of variance explained by the individual pigs concurrent with less residual variance compared to the skin samples (13.1% vs 35.7%) (Table 1), suggesting that nose samples are better suited for reproducible sampling.

Level of MRSA in the 3 farms

A total of 1,120 nasal and skin samples were obtained and the MRSA load was generally found to be highest in the weaning and farrowing sections and lower in the gilt and gestating sow sections. Similar results were obtained from the samples of airborne MRSA. An overview of the detected MRSA loads is presented in Table 2.

In the farm (Farm 2, Fig. 1) where all units were visited, the nasal load of MRSA was highest in the weaning unit followed by the farrowing unit, where the detected load was significantly lower (p<0.001) (Fig. 1). There were, however, no significant difference between these two units regarding the MRSA level of the skin samples (p=0.98) or air samples (p=0.47). In all three sample types, nasal, skin and air samples, the MRSA loads in the weaning and farrowing units were significantly higher than the loads detected in the gilts and gestating sows (p<0.001), where the lowest loads were seen. There was no significant difference in the MRSA loads observed among the gilts and the gestating sows (p>0.05) in any of the sample types.
Air measurements are a better proxy for nasal MRSA levels than for skin levels

There was a significant correlation between the average nose and air levels, (pearsons $r^2=0.38$, p<0.01) with a regression slope of 0.97 (SE±0.08) (Fig. 2). The correlation between the skin and air was not found to be significant (pearsons $r^2=0.05$, p=0.34).

DISCUSSION

The level of MRSA was quantified in a total of 1,120 animal swab samples and 40 air samples obtained from 10 individual sections located at three different farms. This investigation found the level to be significantly higher in the farrowing and weaning units compared to the units with gilts and gestating sows for all three sample types (Table 2, Fig. 1). These findings are in agreement with previously published results, which show an accumulation of MRSA over time, with the highest prevalence seen in weaning pigs, followed by a decrease in the finishing compartment where pigs are of the same age as gilts (Broens et al. 2012).

The increased levels in the farrowing and weaning units could be due to the high number and increased stocking density of pigs per pen in these two section types, compared to gilts and gestating sows. Transmission through direct contact between animals has been shown to be the most important transmission route (Broens et al. 2012). Results from a study of MRSA in organic pigs, show that MRSA on pigs with access to open fields, becomes undetectable within a short period of time, indicating spontaneously clearance (DANMAP 2016). In line with this, a recent study showed a high proportion of pigs categorized as intermittent carriers (Espinosa-Gongora et al. 2015), which was suggested to illustrate a re-contamination of pigs within farms by persistent carriers, caused by the high density of pigs and heavy environmental *S. aureus* contamination. Both studies emphasize on the importance of the housing conditions.
Other important factors, which could contribute to the high level of MRSA found in all weaning sections, could be the usage of antimicrobials (Moodley et al. 2011). In 2016, more than 34 tons of antibiotics were used in weaning pigs, which correspond to 45% of the total consumption of antibiotics used for Danish pigs (DANMAP 2016). Tetracyclines constitute around 30% of the total usage of antibiotics for pigs and tetracyclines are assumed to select for MRSA (Larsen et al. 2016). In line with this, high amounts (approx. 500 tons/year) of zinc oxide are used only in the weaning sections (DANMAP 2016) which is also assumed to select for MRSA (Cavaco et al. 2011).

The increased MRSA levels in the farrowing and weaning sections were found in all three sample types; air, nose and skin (Table 2). However, inconsistency was seen between skin and nasal swabs in the farrowing and weaning sections. It would be interesting to investigate if both anatomical locations are true colonization sites of MRSA or if one (or both) is merely a result of MRSA contamination from the environment.

The high load of MRSA in the air of weaning sections supports the previously suggested bi-directional association of positive pigs and increased amounts of MRSA in the air (Friese et al. 2012). In contrast to our results, Friese et al. (2012) only saw this association when looking at prevalence of positive pigs and not the colony counts of MRSA in nasal swabs.

We found air measurements to be a better proxy of nasal levels than skin levels. The correlation in Figure 2 show that 38% of the nasal load can be explained by the air load (Fig. 2) where as 5% of the skin load was explained by the air load. This observation could be important in relation to interventions against MRSA in farm environment, regarding which samples to obtain for assessment of intervention effect. These results could also be influenced by the sampling method where the amount of sample material could vary as sampling of live pigs can be difficult to standardize (Friese et al. 2012), which could be an limitation of the method. A comparison of the two types of swabs for quantification was
performed to assess which type to be most reproducible, and based on the degree of residual variance, 13% in nasal compared to 35.7% in skin samples, we suggest nasal samples be better suited for reproducible sampling (Table 1). Perhaps the larger proportion of unexplained variation in skin swabs partly could explain the lower correlation between air and skin. In a study where the most probable number method was applied on nasal swabs, linear regression model found that the variation in nasal samples with loads between 0 and 10,000 CFU/swab were not related to the individual pig but to unknown factors of the farm and pen. Variation in samples between 10,000 and 100,000 CFU/swab was shown to be less influenced by farm and pen and significantly influenced by the individual pig (Espinosa-Gongora et al. 2015).

CONCLUSION

The highest levels of MRSA within pig farms were seen in the farrowing and weaning unit which means, that the highest risk for farmers to become colonized with MRSA occurs when working within these two units. Unfortunately, these two units are where the most intense human-pig contact occurs. This makes the farrowing and weaning unit the most important candidates for implementation of intervention strategies, in order to reduce the level of MRSA within farms and to limit the human risk of exposure. Of note, we found nasal swabs to be better suited for reproducible sampling, which is of relevance when following changes in load over time as part of intervention assessment. The amounts of airborne MRSA of sections were found to be a better proxy to determine the level of MRSA in the nose of animals compared to the skin. We do not find this sufficient to recommend that the air load would be enough for complete quantification of MRSA. However, air sampling is an easy and inexpensive method to assess a low or high MRSA status of the section, but it should be emphasized that a negative air sample does not necessarily equal an MRSA negative status for the individual pig and section.
ACKNOWLEDGEMENTS

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DANMAP 2015 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032.

DANMAP 2014 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN 1600-2032.
Table 1. Method assessment. Proportion of the variance originating from different sources, when data was analyzed using a mixed model with a random effect of biological replicates and sampling side nested on pig.

<table>
<thead>
<tr>
<th>Source</th>
<th>Nasal swabs [%]</th>
<th>Skin swabs [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological replicate</td>
<td>25.6</td>
<td>28.7</td>
</tr>
<tr>
<td>Pig</td>
<td>61.3</td>
<td>35.6</td>
</tr>
<tr>
<td>Residual</td>
<td>13.1</td>
<td>35.7</td>
</tr>
</tbody>
</table>
Table 2. Overview of MRSA loads on pigs and in air samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Section</th>
<th>No. of observations</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nose</td>
<td>Gestating sow</td>
<td>65</td>
<td>10 CFU/swab</td>
<td>0-470 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Farrowing</td>
<td>71</td>
<td>180 CFU/swab</td>
<td>0-5200 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Weaning</td>
<td>440</td>
<td>435 CFU/swab</td>
<td>0-50000 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Gilt</td>
<td>34</td>
<td>30 CFU/swab</td>
<td>0-35000 CFU/swab</td>
</tr>
<tr>
<td>Skin</td>
<td>Gestating sow</td>
<td>36</td>
<td>30 CFU/swab</td>
<td>0-3290 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Farrowing</td>
<td>37</td>
<td>200 CFU/swab</td>
<td>5-12500 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Weaning</td>
<td>410</td>
<td>160 CFU/swab</td>
<td>5-260000 CFU/swab</td>
</tr>
<tr>
<td></td>
<td>Gilt</td>
<td>27</td>
<td>30 CFU/swab</td>
<td>5-690 CFU/swab</td>
</tr>
<tr>
<td>Air</td>
<td>Gestating sow</td>
<td>8</td>
<td>8 CFU/m³</td>
<td>4-32 CFU/m³</td>
</tr>
<tr>
<td></td>
<td>Farrowing</td>
<td>8</td>
<td>80 CFU/m³</td>
<td>44-148 CFU/m³</td>
</tr>
<tr>
<td></td>
<td>Weaning</td>
<td>20</td>
<td>4230 CFU/m³</td>
<td>28-8650 CFU/m³</td>
</tr>
<tr>
<td></td>
<td>Gilt</td>
<td>4</td>
<td>2 CFU/m³</td>
<td>2-26 CFU/m³</td>
</tr>
</tbody>
</table>
Figure 1. Detected MRSA loads in the nose of the animals. An overlapping boxplot shows median, first and third quartile for each plot.
Figure 2. Linear association between the MRSA load in nose and air. Intercept is forced through zero.
Low prevalence of MRSA CC398 in dairy cattle and veal calf herds in Denmark – evidence of spillover from pigs

Manuscript in preparation

Introduction to the study

High intensity of animal contact is a risk factor of human acquisition of MRSA CC398. In addition, high within-farm levels of MRSA might increase the risk of spread into the human community and into other animal reservoirs. This spread can occur through human traffic but also via e.g. spread through exhaust farm-ventilation, which possibly can induce spread to other farms in dense rural areas. In the Netherlands, where a similar to the Danish situation of MRSA CC398 in pigs is found, emergence of MRSA CC398 positive veal calves and dairy herds have been observed.

The following study was initiated to investigate a possible spillover of MRSA CC398 from pigs into Danish cattle.
Low prevalence of MRSA CC398 in dairy cattle and veal calf herds in Denmark – evidence of spillover from Danish pigs

Julie E. Hansen\textsuperscript{a}, Troels Ronco\textsuperscript{a}, Marc Stegger\textsuperscript{b}, Raphael Sieber\textsuperscript{b}, Mette E. Fertner\textsuperscript{a}, Henrik L. Martin\textsuperscript{c}, Mikael Farre\textsuperscript{c}, Nils Toft\textsuperscript{a}, Anders R. Larsen\textsuperscript{b}, and Karl Pedersen\textsuperscript{a,*}

\textsuperscript{a}National Veterinary Institute, Technical University of Denmark, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark

\textsuperscript{b}Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark

\textsuperscript{c}SEGES P/S, Agro Food Park 15, DK-8200 Aarhus N, Denmark

Intendend for Veterinary Microbiology

*Corresponding author:

Karl Pedersen
Technical University of Denmark
National Veterinary Institute
Kemitorvet
DK-2800 Kgs. Lyngby
Denmark

E-mail:kape@vet.dtu.dk
Abstract

The possible spillover from pigs into other production animals incites concern for unresolved reservoirs for human exposure. The present investigation was therefore initiated, to elucidate if Danish veal and dairy herds constitute a reservoir of MRSA CC398 and to identify the source of introduction. We collected nasal swab samples from 17 Danish veal farms, 2 slaughter houses and received bulk tank milk samples from 286 dairy herds. All samples were analyzed by culturing and screening on MRSA selective plates and presumed MRSA were verified by MALDI-TOF and PCR. MRSA isolates were subjected to spa typing and whole-genome sequencing. MRSA was found on two veal farms in one and three calves, respectively, with subsequent follow-up samples found negative. Eight of 286 dairy farms (2.8%) were found MRSA positive and follow-up samples, from a subset of farms showed intermittently detection of MRSA in bulk tank milk. Based on spa typing, t034 and t011 was the common spa types, however a single isolate from a dairy herd belonged to spa type t843 associated to mecC-MRSA CC130. Of note, this is the first report of mecC-MRSA in the Danish dairy production. A phylogenetic analysis based on single nucleotide polymorphisms and genomic characterization identified Danish pigs as the original host of the identified isolates, verifying spillover into the veal and dairy herds. Results of the investigation indicated a contamination of veal farms while some dairy farms seemed to be a permanent reservoir. Thus, Danish cattle represent a low prevalence reservoir of MRSA CC398, however, at present, not of major human health concern.

Keywords: Bulk tank milk; veal calves, MRSA, CC398, mecA, mecC, WGS
1. Introduction

The presence of methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC)398 has been reported in a wide range of different food animals from multiple European countries (Cuny et al., 2010). Denmark has experienced a rapid increase of MRSA CC398 in its pig production. A baseline study conducted by the European Food Safety Authority (EFSA) in 2008, found a prevalence of 3.5% in production holdings with breeding pigs and 0% in breeding holdings (EFSA, 2009). However, in 2016, a survey conducted by the Danish Veterinary and Food Administration, found a prevalence of LA-MRSA in randomly selected finisher production herds of 88%, an increase from 68% in 2014 (DANMAP 2014;2016). This development has gained considerable media attention in Denmark due to the simultaneous increase in number of people testing positive for MRSA CC398 (DANMAP 2016). As a result, concerns were raised about the possible spillover of MRSA CC398 from the pig production into other livestock, such as dairy cattle and veal calf production. MRSA CC398 isolates have been detected in different bovine reservoirs reported from multiple countries, and transmission of MRSA to personnel has been described (Fessler et al., 2012; Graveland et al., 2010; Juhász-Kaszanyitzky et al., 2007; Van Cleef et al., 2015; Vandendriessche et al., 2013).

Based on the rapid dissemination of MRSA CC398 throughout the Danish pig production, and the expansion of the animal host range found in other European countries, this study was initiated with the purpose to investigate if Danish veal and dairy herds constitute a reservoir of MRSA CC398 and to possibly identify the source of introduction. For dairy herds, only a single investigation has so far been performed in Denmark. In 2012, 4/219 (1.8%) bulk tank samples were found MRSA positive (DANMAP 2012). However, given the dramatic increase in the prevalence of MRSA CC398 seen in pigs, new information about the level of MRSA CC398 in Danish dairy herds is be relevant.
The possible association to the Danish pig production, of MRSA CC398 isolated from other animals than pigs, can be established with WGS and phylogenetic analysis based on single nucleotide polymorphisms (SNPs). In Denmark, three different lineages, L1-L3 are known to dominate (Sieber et al. unpublished data). Certain genomic markers, such as \textit{tetM}, \textit{tetK} and \textit{czrC}, are indicators of pig-association and genes related to the human immune evasion cluster (IEC) are typically found in the isolates of basal human origin (McCarthy et al., 2012; Price et al., 2012). The presence or absence of these marker genes can also aid in the identification of the origin of a given CC398 MRSA isolate.

2. Materials and methods

2.1. Pilot study

As a pilot study, it was decided to sample calves at slaughter to a) estimate the number of MRSA positive animals for later on-farm sampling; b) determine the optimal sampling location on the animal by swapping the nostrils, groin and perianal region. A total of 93 calves were sampled at slaughter during two sampling rounds at an abattoir. The 93 calves originated from 15 different veal calf holdings who received their calves from 45 different dairy cattle farms.

2.2. Sample collection

Nasal swabs from 620 veal calves were collected on farm from 17 different farm holdings, a minimum of 25 samples per farm. The farms were selected to geographically represent all of Denmark, and all farm holdings included in this study were large production holdings with 300-2000 animals on site.

Bulk tank milk (BTM) samples were obtained during multiple collection rounds. In 2014, samples were obtained from 50 dairy herds randomly selected among the Danish milk producers. Follow-up samples were collected at 10 different time points, from five dairy herds associated by a common
owner, from which one herd was included in the initial screening of 50 herds. In 2015, a total of 236 samples were received for screening collected and submitted by Eurofins. Of these, 108 samples originated from farms selected on the basis of previously established low Ct-value for S. aureus and 128 originated from farms selected based on previously established low Ct-value for S. agalactiae.

2.3. Sample processing and MRSA identification

Swabs were enriched in 5 ml Mueller-Hinton broth supplemented with 6.5% NaCl for 18-24 h of incubation at 37°C. A 10 µl loopful of broth was cultivated on Brilliance MRSA 2 agar plates (Oxoid, UK). A subset of samples (n=280) were additionally cultivated on Brilliance Staph 24 agar (Oxoid, UK) followed by incubation for 24 h at 37°C. One presumptive MRSA/MSSA colony from MRSA 2 and Staph 24, respectively, were sub-cultivated onto blood agar. Isolates were identified as S. aureus by MALDI-TOF (Bruker, Bremen, Germany). Multiplex PCR was performed to detect mecA and nuc (Maes et al., 2002) and an additional PCR detecting mecC (García-Álvarez et al., 2011), if mecA negative in duplex PCR. Isolates were stored at -80°C. One mL of each sample containing BTM was enriched 18-24 h at 37°C in 9 mL Mueller-Hinton broth supplemented with 6.5% NaCl, and subsequently streaked onto Brilliance MRSA2 agar (Oxoid). Presumptive MRSA colonies were treated as stated above.

Spa typing of all MRSA isolates were performed as previously described (Mellmann et al., 2006; Stegger et al., 2012).

2.4. Strain collection for whole-genome sequencing

A total of 19 MRSA isolates from the screening of dairy (n=15) and veal calf (n=4) herds were available for sequencing. In 2016, an aseptic foremilk sample was collected from a Danish dairy cow with clinical mastitis according to the National Mastitis Council’s guidelines, from which MRSA strain Sa52 was derived (Ronco et al., 2017). The mastitis isolate was included in the
genomic characterization and phylogenetic analysis. Isolates of MRSA CC398 from Danish pigs (n=183) previously described by Sieber et al. (unpublished data) was included for comparison and additionally 88 international isolates previously described by Price et al. (2012) were included as a reference collection.

2.5. Strain preparation and whole-genome sequencing

A total of 20 isolates (clinical mastitis isolate=1, veal calves=4, BTM=15) were subjected to whole-genome sequencing. The DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) were used to extract DNA according to manufacturer instruction, except that a pretreatment with lysostaphin was applied before extraction. The library preparation was performed using an Illumina Nextera XT kit (Illumina Inc., USA) and run on Illumina’s NextSeq instrument (Illumina Inc., USA) for 150 bp paired-end sequencing. Whole-genome sequencing data for the international reference collection (Price et al. 2012) were obtained from the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) via BioProject accession number PRJNA274898. The Danish porcine strains were obtained from the Sieber et al. (unpublished data).

2.6. Single nucleotide polymorphism (SNP) calling and phylogenetic analysis

Using NASP (Sahl et al., 2016), identification of SNP variants was performed using the GATK UnifiedGenotyper with filtering set to remove positions with less than 10 fold coverage and 90% unambiguous variant calls after positions within duplicated regions of the reference sequence was removed using NUCmer. Purging of the 123 kb recombinant region of the ST398ST9 hybrids was performed prior to phylogenetic reconstruction using FastTree 2.1.5 as implemented in Geneious 11.0.2 (Biomatters, Auckland, New Zealand).
2.7. Genomic characterization of isolates

The presence of resistance determinants was assessed using raw sequence reads in Mykrobe Predictor version 0.4.3 (Bradley et al., 2015). Using the same software, the genotype of additional genes of interest was determined with results filtered for >80% coverage and >5 median depth. The reference sequences were obtained from NCBI genomes with accession number DQ530361 for Sa3int, sak, scn and sea, BA000018.3 for sep, KF593809.1 for the cadmium-zinc resistance gene czrC and NC_013450 for genes SAAV_2008 and SAAV_2009 associated with virulence in avian hosts.

3. Results

3.1. Pilot study

No MRSA was found in any of the samples taken from calves at slaughter, indicating that if present, it would be in a very low prevalence. It was decided to sample at least 25 individuals on each farm depending on the farm layout, to achieve at least the same within herd sensitivity as in the Danish screening of pig farms published in DANMAP 2014. No new information was obtained regarding optimal body location of sampling, and the nostrils were chosen as sampling location for further samplings, as it is the most used sampling site when screening for MRSA.

3.2. Occurrence of MRSA in veal calves and bulk tank mink

Two of the 17 (11.8%) farms had at least one positive calf. On the positive farms, one and three calves were found positive, respectively. All four isolates were typed as spa type t034 associated to CC398. In a follow-up investigation (1½ month later), the four calves and their penmates were negative. A total of 286 BTM samples were screened for the presence of MRSA and eight samples were found to be MRSA positive corresponding to 3%. Seven of the eight isolates were mecA positive and of spa type t034 belonging to CC398 and a single isolate was mecC positive was of
spa type t843 associated to mecC-MRSA CC130. The mecC-MRSA was excluded from the phylogenetic analysis and further genomic characterization. One of the positive samples was from a farm with an owner who had four additional dairy cattle farms, where farm workers and animals were moved between farms. Hence, follow up samples were taken at all 5 farms. Samples were collected repeatedly during nine months, however not all farms were sampled at every time point, resulting in a total of 40 samples, which were investigated for MRSA. Ten of these samples contained MRSA, with three farms intermittently found positive and two farms never found positive. Nine of the detected isolates belonged to spa type t011 (repeat succession: 08-16-02-25-34-24-25) associated to CC398 and a single isolate belonged to a new spa type, t15971 (repeat succession: 08-16-02-25-34-24-25-02-25-34-24-25), which could have resulted from a single genetic event with the duplication of five repeats 02-25-34-24-25. Nine of the detected isolates were available for sequencing and further genetic characterization.

3.3. Genomic characterization of the isolates

Isolates from the clinical mastitis case (n=1), veal calves (n=4) and BTM (n=15) were characterized on the basis of genomic identification of resistance genes and additional genes associated to different host. None of the isolates were found to carry any of the human related IEC genes (Sa3int, sak, scn, sea and sep) or the genes related to avian-host-specificity SAAV_2008 and SAAV_2009. All 20 isolates were found to carry mecA, tetM and blaZ, whereas dfrG was detected in 19/20 (95%) of the isolates, but absent in the mastitis strain Sa52. Strain Sa52, the four isolates from veal calves and six of the BTM isolates were found to carry the gene encoding cadmium-zinc resistance, czrC, however the nine isolates originating from the three common owner dairy farms were found not to carry czrC. The lnuB gene was found in 9/20 (45%) isolates and two genes encoding erythromycin resistance, ermA and ermB were detected only in the mastitis isolate.
3.4. Phylogenetic analysis

Based on the phylogenetic relationship seen in Figure 1, 18/20 bovine isolates were found to cluster within the three dominating lineages, L1-L3, present in Danish pigs. The nine BTM isolates from the three farms with a common owner were found to cluster within L1. All but three of the remaining isolates were found in the L3-clade, whereas the strain Sa52 was found in the L2-clade and two BTM isolates were found outside of the dominant pig lineages.

4. Discussion

We found MRSA CC398 in two of 17 Danish veal farms. In the two positive herds however, no evidence of persistent MRSA CC398 colonization in veal calves was found. In Danish dairy cattle we found MRSA CC398 in BTM from eight of 286 dairy herds. Repeated sampling of BTM in one of the positive farm and four contact herds showed intermittent detection of MRSA CC398. Based on these findings, we conclude that MRSA CC398 was present in the Danish dairy and veal calf production, however in considerably lower frequencies than observed in the Danish pig production (DANMAP 2016).

The most prevalent spa types were t034 and t011 which is in accordance to previous findings, where MRSA CC398 t011 was found in Danish retail beef, and t011 and t034 in BTM (Agersø et al., 2012; DANMAP 2012). Both spa types are recognized as the most common spa types in the Danish pig production (DANMAP 2014).

The identified spa types and the low prevalence of MRSA CC398 positive veal and dairy farms could indicate spillover from the pig production. Tavakol et al. (2012) previously provided evidence of transmission of MRSA CC398 from pigs to cattle, and Locatelli et al. (2016) illustrated an exposure-response relationship between the MRSA positive status of dairy farms and the number of surrounding swine and swine herds. Based on the phylogenetic analysis (Figure 1), it is evident that the isolates found in the different bovine sources were of the same lineages as what is
found in the Danish pig production, confirming the presence of MRSA CC398 as a spillover from Danish pigs into the Danish dairy and veal calf production.

The four veal calf isolates and four BTM isolates found in L3 had identical resistance patterns, whereas the two BTM isolates found outside of the dominant pig-lineages, did not carry the additional tetracycline resistance gene, tetK, characteristic for pig-associated MRSA CC398. The loss of tetK has previously been observed in isolates removed from the selective pressure of tetracycline in the pig environment. It is possible that these two isolates did not come directly from pigs, but have been introduced into the dairy herds after a period of absence from the farm environment, while occupying another host e.g. humans. Human introduction is not atypical as observed in Norway (Grøntvedt et al., 2016). However, the presence of tetM and dfrG in the two isolates indicate pigs as the origin of the strains, as both genes are indicators of association to the pig-clade (McCarthy et al., 2012). The strain from clinical mastitis Sa52 was the only isolate which encoded erythromycin resistance genes, ermA and ermB, and lacked dfrG. Erythromycin resistance is more commonly found in pig isolates in L2 compared to L1 and L3 (Sieber et al. unpublished data) which is in agreement with the location of Sa52 within L2 as the only bovine isolate. The BTM isolates from the three dairy farms with a common owner were found to be closely related in the phylogenetic analysis, and contained identical accessory gene. Interestingly, all isolates lacked czrC, a gene associated to pig-adaption (Price et al., 2012), which could be a result of shift in niche from pigs to dairy cattle where absence of selective pressure triggered the loss of the cadmium-zinc resistance gene. The intermittent detection of isolates from the three farms indicate low prevalence close to the detection limit, which could be a result of sub-clinical mastitis in the herd as observed in Belgium (Vanderhaeghen et al., 2010), and low within herd prevalence of intra-mammary infections in dairy cows caused by MRSA CC398 has previously been reported (Luini et al., 2015; Spohr et al., 2011). The first clinical mastitis isolate from Denmark, included in the present study, supports the possible problematic development and emergence of MRSA CC398 in Danish dairy
herds that are able to cause mastitis. In 2012, a study using the same methodology as the present study, found a similar low prevalence and intermittent detection of MRSA CC398 in Danish BTM (DANMAP 2012).

The first mecC-MRSA isolate from a Danish dairy herd was identified in the present study. mecC, was first detected in isolates from bovine milk samples (García-Álvarez et al., 2011) and a Danish study from Petersen et al. (2013) concluded that ruminants may be healthy carriers of CC130 mecC-MRSA and documented transmission in a single case from a cow to its owner. The detection of CC130 mecC-MRSA in BTM as part of the present study, conducted 3-4 years later, support this conclusion, however indicates that mecC-MRSA lineage in cattle has not, at this point, widespread in the cattle production with similar success as MRSA CC398 have in the pig production.

The present study in veal calf holdings is based on a relatively low number of screened farms. We found that MRSA CC398 is detectable in the Danish cattle production, but to assess the actual prevalence with higher precision more herds should be included in a screening. We expected a low prevalence of MRSA-positive farms based on the pilot study at an abattoir. However, if present at a given farm, we did not necessarily expect low within-herd prevalence. This assumption was based on experiences from MRSA-positive pig holdings, but was proved to be incorrect. In future screenings, the number of animals at cattle holdings should be increased in order to increase the sensitivity of detecting MRSA.

5. Conclusion

Results obtained in this study show MRSA CC398 to be present in the Danish dairy and veal calf herds. However, the data indicate that cattle not is an independent reservoir of MRSA CC398, as the positive finding may rather be a result of contamination from e.g. farm workers as observed in Norway (Grøntvedt et al., 2016). The phylogenetic analyses verify a spillover from pig production into cattle production. Based on the genomic characterization, it seemed that the veal herds and
some dairy herds were merely contaminated, while persistent low presence of MRSA CC398 in
some dairy herds was observed. Of note, mecC-MRSA was for the first time identified in a Danish
dairy herd. The few detected cases in dairy herds and veal calf herds, indicate that the cattle
production does not represent a substantial reservoir of MRSA CC398 of human health concern at
this point. However, lessons from the spread in the Netherlands in veal calves and dairy cattle as
well as the Danish experience with CC398 in swine, may emphasize the need to routinely conduct
similar investigations to survey if any changes develop over time.

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Figure 1. Phylogenetic relationship of MRSA CC398 from bulk tank milk, veal calves, pigs and from a worldwide reference collection. The phylogeny includes 20 isolates from multiple bovine sources (bulk tank milk: n=15, red and pink dots; veal calves, n=4, grey dots; clinical mastitis, n=1, green dot). Bulk tank milk is divided in red dots which correspond to isolates from three farms with common owner and pink dots correspond to unrelated farms. A total of 183 isolates related to Danish pigs from 2014 (Sieber et al. unpublished data) seen as black dots and 89 international isolates included as reference phylogeny from Price et al. (2012) seen as grey nodes. Dominating pig lineages are seen in L1=dark grey, L2=black and L3=light grey. The tree has been rooted according to Price et al. (2012).
Livestock-associated methicillin-resistant *Staphylococcus aureus* is widespread in farmed mink (*Neovison vison*)


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**Introduction to the study**

Denmark has the largest production of pelted skins in the world, and many people are employed in the Danish production of mink. Indications based on infections of MRSA CC398 detected in people with contact to mink and identification of MRSA CC398 from autopsies of diseased mink, point to mink as a possible newly emerged reservoir of MRSA CC398.

The following study was therefore initiated in order to assess the degree of LA-MRSA in mink and to determine if mink do represent a reservoir of MRSA CC398.
Livestock-associated methicillin-resistant Staphylococcus aureus is widespread in farmed mink (Neovison vison)

Julie Elvekjær Hansen⁎, Anders Rhod Larsenb, Robert Leo Skovb, Mariann Chriël, Gitte Larsena, Øystein Angenb, Jesper Larsenb, Desireé Corvera Kløve Lassenab, Karl Pedersena

a National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark
b Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark

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ABSTRACT

Livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) clonal complex (CC) 398 is widespread in the Danish pig production with around 90% of herds being positive. Since 2009, cases of CC398 LA-MRSA infections in Danish mink farmers have been observed. The objective of the study was to examine the presence of LA-MRSA in farmed mink.

The investigation comprised three different sample types 1) clinical samples from carcasses submitted to the laboratory for diagnostic examination, 2) paws and pharyngeal swabs from healthy animals collected at pelting, and 3) feed samples from mink feed producers.

In clinical samples, LA-MRSA was found in 34% of submissions and was most prevalent in samples from paws (33%) and pharynx (17%), followed by nasal and intestinal samples (each 13%), whereas it was never detected in perineal samples. LA-MRSA was found in healthy animals on 40% of the investigated farms, including paw samples (29%) and pharyngeal samples (16%). Twenty out of the 108 feed samples from feed producers were positive for LA-MRSA. The dominant spa-types were t034 and t011 associated to CC398, corresponding to the dominant spa-types detected in the Danish pig production, from which slaughter offal is used for mink feed. The spa-types, the high prevalence of LA-MRSA on paws and in pharynx, and its detection in feed samples, suggest feed as a possible source of LA-MRSA in mink.

1. Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a major concern in human medicine (Kluuytmans and Struelens, 2009; Cuny et al., 2012; DANMAP, 2015). Primarily, MRSA emerged in the healthcare systems during the 1960s, but later spread into the general society. In recent years, MRSA has furthermore emerged among farm animals, the so called livestock-associated MRSA (LA-MRSA) (Fitzgerald, 2012; Graveland et al., 2011; Price et al., 2012). LA-MRSA has especially been found in pigs but also in several other food-producing animal species, in companion animals, and in wildlife (Cuny et al., 2015, 2010).

In Europe, clonal complex (CC) 398 is the dominant LA-MRSA genotype. LA-MRSA is widespread in the Danish pig population with a herd prevalence of around 90% in 2016, whereas this bacterium is only found at a very low frequency in other food-producing animal species (Miljø- og fødevarestyrelsen, 2017; DANMAP, 2015; Hansen et al., 2016). In Denmark, the first cases were reported in humans in 2004 (Larsen et al., 2015). Since then, the number of cases has been rapidly increasing and LA-MRSA is now the predominant type of MRSA in Denmark (DANMAP, 2015). Most people carrying MRSA CC398 have direct contact to livestock or are household members to people with livestock contact.

LA-MRSA belonging to CC398 was first detected in mink in clinical samples submitted to the National Veterinary Institute (DTU-VET) during 2013 (Larsen et al., 2016a), but from then on the bacterium has been found in several samples from mink investigated at DTU-VET. In addition, a total of 65 human LA-MRSA cases reported contact to mink between 2009 and 2015 (A.R. Larsen, unpublished data). The mink industry is very large in Denmark. In 2016, 17.1 mill pelted skins were produced in Denmark out of the world production of 55.8 mill (Kopenhagenfur, 2017). More than 6000 Danes work in the mink industry and are potentially exposed to LA-MRSA (Danske minkavlere, n.d.).
The objective of the present study was to determine the degree of LA-MRSA carriage in mink. We address the presence of LA-MRSA in mink and discuss feed as a potential origin of infection and the impact of LA-MRSA on the human working environment. The investigation was carried out on clinical material submitted for autopsy, as a survey of healthy animals and feed swabs collected on farms, and on feed samples from mink feed producers.

2. Materials and methods

Different investigations have been carried out during this study as seen in Fig. 1 which illustrates sample collections, sample types, and sample processing.

2.1. Sample collection

2.1.1. Clinical submissions

During 2015, clinical submissions from 58 individual mink farms were investigated for MRSA. From the first 36 submissions the nose, pharynx, perineum, intestine (5 cm jejunum), and the right forepaw (cut at the carpal joint) were investigated. From the remaining 22 submissions only the pharynx and right forepaw were investigated. Samples from similar anatomical sites in up to five animals from each submission were pooled. If more than five animals were submitted per farm, material was pooled in two sets of even pools.

2.1.2. Screening of mink farms

During November and December 2015, the Danish Veterinary and Food Administration conducted a screening survey of feed and healthy animals on 50 mink farms. The samples were taken from the animals during pelting time. From each farm, 5 pools of pharyngeal swabs from 5 mink each (25 animals) and 2 pools of 5 paws (10 animals) were collected immediately after pelting. Additionally, 2 pools of 5 feed swabs from feeding areas (10 samples) were collected at each farm.

2.1.3. Screening of feed samples from mink feed producers

During 2016, a total of 108 feed samples were received for analysis. The samples were collected from a total of 14 mink feed producers covering all of Denmark, and were sent to DTU-VET from a laboratory conducting multiple analyses of animal feed samples.

2.2. Sample processing

2.2.1. Clinical submissions

Swab samples were enriched in 3 ml Mueller- Hinton broth (MHB) (Oxoid, Basingstoke, UK) supplemented with 6.5% NaCl (MHB+), whereas intestinal sample material and the sample of paws were weighted and added a ten-fold amount of MHB+. All sample types were incubated at 37 °C for 18–24 h with agitation at 180 rpm. This procedure will be referred to as one-step enrichment.

2.2.2. Screening of mink farms

Both pharyngeal and feed swabs were first pre-enriched in MHB+, followed by a second enrichment where 1 ml MHB+ broth was transferred to 9 ml Tryptone Soy Broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 3.5 mg/l cefoxitin and 20 mg/l aztreonam (supplemented TSB) and incubated at 37 °C for 18–24 h without agitation. This procedure will be referred to as two-step enrichment. The paw samples were analyzed using one-step enrichment.

2.2.3. Screening of feed samples from mink feed producers

The feed samples were kept frozen until analysis. All samples were analyzed by one-step enrichment, where 10 g were inoculated in 90 ml MHB+ for 18–24 h at 37°C without agitation.

2.3. MRSA identification of all sample types

A loopful (10 μl) enriched sample material was streaked on Brilliance MRSA2 agar (Oxoid, Basingstoke, UK) and incubated at 37 °C for 18–24 h. Presumptive MRSA colonies identified as denin blue colonies on MRSA2 agar were sub-cultured on agar plates (Oxoid, Basingstoke, UK) containing 5% calf blood for further verification. Isolates were identified as MRSA by PCR detection of the meCA and nuc genes (Maes et al., 2002). spa-typing was performed as previously described (Mellmann et al., 2006; Stegger et al., 2012).
3. Results

3.1. Clinical submissions

Twenty of the 58 clinical submissions were positive for LA-MRSA (34%) in one or more sample types (Table 1, Fig. 2A). LA-MRSA was most frequently found on the paws (33%) followed by the pharynx (17%). Out of the first 36 submissions, the nose and intestine were found positive in 13%, whereas none of the perineal samples were positive (Table 1). Investigation of the first 36 submissions showed that in cases with a positive intestinal or nasal swab sample, a paw and/or a pharyngeal swab sample were also invariably positive. Nasal samples, intestinal samples, and perineal samples did therefore not add to the sensitivity of LA-MRSA detection in mink, and was omitted in the remaining 22 clinical submissions.

3.2. Screening of mink farms

Twenty out of the 50 screened mink farms (40%) were found positive for LA-MRSA in one or more of the samples (Table 2, Fig. 2B–D). LA-MRSA was most frequently found on the paws (29%) followed by pharyngeal samples (16%) and feed samples (8%).

3.3. Screening of mink feed producers

A total of 19% (20/108) of the investigated feed samples obtained from feed producers were found LA-MRSA positive.

3.4. spa-types

spa-typing from all three investigations, showed a dominance of t034 (57%) followed by t011 (30%) in addition to a few isolates of spa-type e571 (6%) and r2876 (2%).

3.5. Antimicrobial susceptibility testing

As expected, all isolates were resistant to beta-lactam antibiotics, (i.e. penicillin and cefoxitin) and tetracycline, but high levels of resistance were also recorded to spectinomycin, streptomycin, erythromycin (representative of macrolides), trimethoprim, and tiamulin. Intermediate level of resistance to sulphanomides was recorded. Low levels of resistance were recorded to chloramphenicol, gentamicin, and ciprofloxacin (representative of fluoroquinolones). No resistance was recorded towards florfenicol and potentiated sulphonamides (Table 4).

4. Discussion

To our knowledge, this is the first report on LA-MRSA in mink. We show that LA-MRSA is prevalent on Danish mink farms.

The presence of coagulase-positive staphylococcal species in mink has previously been investigated. In a study by Guardabassi et al. (2012), mink was found to be natural host of S. delphini group A, but not S. aureus. In 1995, the first cases of Staphylococcus intermedius were described in mink by Hesselbarth and Schwarz (1995), and Pedersen et al. (2009) described hemolytic staphylococci as one of the most important reasons for infectious disease in mink, most often caused by S. intermedius and occasionally S. aureus. As there was no previous information on the biology of LA-MRSA in mink, we investigated in which anatomical sites LA-MRSA was located in the animals. The nose of the mink was considerably less often positive for LA-MRSA than both the paws and the pharynx. This is important information concerning relevant sampling sites in animals. Most investigations for MRSA in various animal species have used nasal swab samples or clinical material (Walther et al., 2008; Wardyn et al., 2012).

The widespread presence of LA-MRSA on mink farms could be due to usage of antibiotics, however the prevalence of LA-MRSA in clinical submissions and healthy mink were similar. Based on the experiences in a Norwegian study, human introduction could be suspected, a possible route of introduction which deserved focus in all production animals (Gruntvæd et al., 2016). However, in the present study an introduction via feed is strongly suspected. The presence of LA-MRSA on the paws of the mink, but absence in the perineal area may suggest that the mink were not colonized but rather contaminated from environmental sources, such as the feed. This is corroborated by the finding of LA-MRSA in both pharynx and intestinal samples, and the fact that feed swab samples at farms and 20 out of 108 mink feed samples from feed producers were positive for LA-MRSA. However, it is necessary to characterize the isolates of LA-MRSA from mink feed and animals further, e.g. using whole genome sequencing, in order to verify feed as a possible route of introduction of LA-MRSA into mink farms.

Mink feed contains slaughter offal from the pig and poultry industry, and provided that a proper heat treatment has not been carried out, there is a risk of transferring LA-MRSA or other pathogenic organisms to the mink. In accordance, the two spa-types most prevalent in mink, t034 and t011 associated to CC398, are the same as those being most prevalent in Danish pigs (DANMAP, 2015). In some cases, more than one spa-type could be detected in samples from a single farm which supports continuous and multiple introductions. Mink feed has previously been associated with outbreaks of disease in mink due to insufficient heat treatment, such as Salmonella Dublin (Dietz et al., 2006), Clostridium septicum (Larsen et al., 2016b) or influenza virus (Gagnon et al., 2009). The detected spa-types belong to the predominant spa-types among MRSA CC398, but the discriminatory power of spa-typing is insufficient to determine the transmission routes. Further studies of extensive genotyping, are now in progress to clarify the importance of feed as a potential source of LA-MRSA in mink.

In the present study, all mink isolates were resistant towards tetracycline and 43% were resistant to erythromycin, whereas all isolates were sensitive to the combination of trimethoprim-sulphonamides. Resistance towards the mentioned antibiotics were similar to what is observed in isolates from human MRSA CC398 cases in Denmark (DANMAP, 2015). These results point to a common source of LA-MRSA in mink and humans, which further support spillover of LA-MRSA from pigs to mink. However, as similar resistance patterns have been recorded also from other sources (Fejler et al., 2010), further genotyping investigations are, as previously mentioned necessary.
LA-MRSA in production animals is primarily a working environmental problem which constitutes a risk of human exposure. In pig farms infected with LA-MRSA, the experience is that most of the pigs in the farrowing and weaning sections are colonized with LA-MRSA in the nose and/or the skin, and there will be high numbers of LA-MRSA in air and dust (Hansen et al., 2015). Accordingly, one risk of human exposure in the pig production is merely from entering the stable (Bos et al., 2016). Whether this is also the case on mink farms is unknown, but it would be relevant to investigate. Differences between pig and mink farming, such as housing conditions, could affect the transmission risk through air, as mink is kept in open air areas compared to conventional pigs housed exclusively indoors. Other risks of exposure to humans is from handling of feed and from bites or scratches when handling animals, e.g. in connection with medication, mating, moving animals, or pelting. Therefore, it is unfortunate that exactly paws and oral cavity are the two body sites where LA-MRSA is most often present.

Fig. 2. Mapped locations of sampled Danish farms. Open circle = MRSA negative sample, closed circle = MRSA positive sample. Framed island represents Bornholm. A) Farms from which clinical submissions originated. B) Paw samples from on farm screening. C) Pharyngeal swab samples from on farm screening. D) Feed swab samples from on farm screening.

Table 2
MRSA in sample types from on farm sampling.

<table>
<thead>
<tr>
<th>Samples per farm</th>
<th>Farms (n = 50*)</th>
<th>No. MRSA positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharyngeal swabs</td>
<td>5 pools from 5 mink</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>Paws</td>
<td>2 pools from 5 mink</td>
<td>14 (29%)</td>
</tr>
<tr>
<td>Feed samples</td>
<td>2 pools from 5 feed swabs</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Total</td>
<td>9 samples</td>
<td>20** (40%)</td>
</tr>
</tbody>
</table>

*Paws only tested from 48 farms.
**Represent no. of MRSA positive farms based on samples of any kind.

Table 3
Combination of pos./neg. sample types from screening of 50 mink farms.

<table>
<thead>
<tr>
<th>Finding of MRSA in different samples</th>
<th>Number of farms with the actual combination of factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharyngeal swabs</td>
<td>Paws</td>
</tr>
<tr>
<td>– – –</td>
<td>30</td>
</tr>
<tr>
<td>– – +</td>
<td>8</td>
</tr>
<tr>
<td>– + –</td>
<td>4</td>
</tr>
<tr>
<td>+ – –</td>
<td>3</td>
</tr>
<tr>
<td>– + +</td>
<td>2</td>
</tr>
<tr>
<td>+ + +</td>
<td>1</td>
</tr>
<tr>
<td>– nd* –</td>
<td>1</td>
</tr>
<tr>
<td>– – +</td>
<td>1</td>
</tr>
</tbody>
</table>

*nd: not determined – paw samples missing from 2 of the 50 screened farms.
This study provides knowledge on farmed mink as a newly emerging reservoir of LA-MRSA and countries with mink productions should be aware of this not previously recognized risk of human exposure.

At present, there is no knowledge regarding the within-herd prevalence of LA-MRSA positive mink on positive farms, due to the use of pooled samples, and due to the very limited number of samples that has been investigated until now. Further, we lack knowledge concerning the persistence of LA-MRSA in mink and mink farms. If feed is the source, it may entirely be a contamination rather than colonization. Further studies are required to clarify these matters.

5. Conclusions

The present investigation clearly demonstrates that LA-MRSA is widespread on Danish mink farms with LA-MRSA found on 40% of the tested farms. The animals most often carry the bacteria on paws and in the pharynx. The dominant spa-type is t034 followed by t011, associated to CC398, similar to what is found in pigs. These observations together with the detection of LA-MRSA in mink feed samples suggest feed-borne transmission. The within-herd prevalence is currently unknown. Humans may be exposed during handling of feed and mink, e.g. in connection with matting or pelting.

Acknowledgements

This investigation was supported by grants from the Pelsdyrægtsfonden, the Danish Veterinary and Food Administration and a grant from The Danish Agrifish Agency (grant no. 33010-NIFA-14-612). The technical assistance of Ms. Margrethe Carlsen, Ms. Alexandra Medina and Ms. Lone Ryste Hansen Klivdevang is gratefully acknowledged.

References


Whole-genome sequencing identifies pigs as source of MRSA CC398 in farmed mink (*Neovison vison*) and mink farm workers


Manuscript in preparation

**Introduction to the study**

MRSA CC389 was found to be present in approx. one third of the tested mink farms included in the previous study (Manuscript number 3). Accordingly, the 6,000 humans employed in the Danish mink production are at possible risk of becoming carriers of MRSA CC398, an issue which has not previously been recognized. The following study was initiated to investigate the emergence of MRSA CC398 in mink and the origin of human cases with reported contact to mink.
Whole-genome sequencing identifies pigs as source of MRSA CC398 in farmed mink

(Neovison vison) and mink farm workers

Julie Elvekjær Hansen⁹, Marc Stegger⁸, Karl Pedersen⁹, Raphael Sieber⁸, Jesper Larsen⁸, Gitte Larsen⁹, Berit Lilje⁸, Mariann Chriél⁹, Paal Skytt Andersen⁸, Anders Rhod Larsen⁸,*

⁹National Veterinary Institute, Technical University of Denmark, Kemitorvet, DK-2800 Lyngby, Denmark

⁸Bacteria, Fungi and Parasites, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark

Intended for Emerging Infectious Diseases

*Corresponding author:
Anders Rhod Larsen
Statens Serum Institute
Department of Bacteria, Fungi and Parasites
Artillerivej 5
DK-2300 Copenhagen S
Denmark
E-mail: ARL@ssi.dk
Abstract

More than 55 million mink skins from Neovison vison are produced globally annually, with the largest production found in Denmark (31%) followed by China, the Netherlands and Poland. Therefore, a large number of people worldwide are employed in mink production. Farmed mink in Denmark were in a recent study found to constitute a reservoir of methicillin-resistant Staphylococcus aureus of clonal complex 398 (MRSA CC398), which previously has been described as the predominant livestock associated (LA-)MRSA lineage in pigs and cattle. The 6,000 mink-related farm workers in Denmark are thus potentially exposed to MRSA CC398 and cases of MRSA CC398 in mink farmers have been reported since 2009. The purpose of this study was to elucidate the source of MRSA CC398 in mink farms and to investigate the possible transmission to the eighty human LA-MRSA CC398 cases with reported contact to mink from 2011-2016. In total, 162 MRSA CC398 isolates originating from mink (n=65), mink feed (n=17) and humans (n=80) with connection to mink, were subjected to whole-genome sequencing, and compared to 183 isolates from Danish pig farms. Genetic characterization of the isolates, including resistance genes and multiple host associated genetic markers did not indicate any specific adaption of MRSA CC398 to mink compared to isolates from pigs or humans. Our study identified MRSA CC398 found in the pig production as the source of MRSA CC398 in mink and mink farmers, and probably with an introduction via the feed. Hindering the continuous introduction of MRSA CC398 into the mink farms could likely eliminate mink as a reservoir for MRSA CC398 and thereby also transmission to mink farmers.
Introduction

Livestock-associated (LA-) methicillin-resistant *Staphylococcus aureus* (MRSA) is prevalent in multiple animal species, with pigs being the predominant host worldwide (Cuny et al. 2010; Petinaki and Spiliopoulou 2012; Fitzgerald 2012). LA-MRSA in pigs is dominated by a few genetic lineages with clonal complex (CC)398 being widely disseminated in Europe. In Denmark, MRSA CC398 prevalence in pigs has increased from 3% in 2008 to 88% in 2016 (DANMAP 2016).

Concurrently, an increase in human cases with or without reported contact to livestock has been observed, showing a spill-over from the pig production to the community and consequently causing disease in humans (Larsen et al. 2017; DANMAP 2016). The CC398 lineage has adapted to pigs and livestock production environment by acquisition of the SCCmec Vc element encoding methicillin, zinc and enhanced tetracycline resistance (*meca*, *czrC* and *tetK*) and by loss of φSa3 carrying the human immune evasion cluster (IEC) (Price et al. 2012). A recent study (Sieber et al. unpublished data) analyzed the population structure of MRSA CC398 in Danish pig production and found that three distinct lineages predominates. The prevalent Danish pig-lineages have recently been observed in Danish horses (Islam et al. 2017) indicating a spillover from the pig production.

It has been shown that mink production constitutes a new animal reservoir of MRSA CC398 in Denmark, where approximately one third of the tested farms were positive (Hansen et al. 2017). Mink are fed with fresh moist feed including slaughter products, amongst other, which e.g. can contain poultry from Germany¹ and liver or whole-blood from Danish pigs². Denmark is the largest producer of mink worldwide, producing 31% (17.1 mill) of all pelted skins, followed by China, the

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It is estimated that more than 6,000 people are currently employed in the Danish mink industry and thus potentially exposed to MRSA CC398. At the National Reference Laboratory for humans at Statens Serum Institut (SSI), national surveillance of MRSA has been ongoing since 1999. A revision of the MRSA guidelines in 2012 included having pig contact as a risk factor for MRSA carriage and in 2016 mink contact was included, too. Humans exposed for these risk factors will be screened for MRSA carriage at admission to hospitals.

The purpose of this study was to evaluate the origin of the human isolates by using whole-genome sequencing of all human MRSA cases in the national MRSA database reporting having mink contact. The isolates obtained from mink and mink feed were also whole-genome sequenced and compared to isolates of human and pig origin to investigate (I) the emergence of MRSA CC398 in mink production and (II) the spread to humans with mink contact.

MATERIALS AND METHODS

Strain collection

This study included MRSA isolates from mink, mink feed, pigs and humans from Denmark. The isolates from mink (n=65) originated from a screening of MRSA conducted in Danish mink farms in 2015 (Hansen et al. 2017) and MRSA from diagnostic mink submissions isolated at the Danish Veterinary Institute from 2014-2016. In addition, 17 of 20 isolates of MRSA from mink feed described by Hansen et al. (2017) were available for sequencing. The human isolates in this study were selected by reviewing the national MRSA database at Statens Serum Institut. Human isolates

2 Danske minkavlere. Available online at: http://www.danskeminkavlere.dk/fakta-om-branchen/
3 The Danish Health Authority available online at: https://www.sst.dk/da/sygdom-og-behandling/smitsomme-sygdomme/-/-/media/F3F52EC1C6A94C6080F50F435DA02E59.ashx
from this collection were included if any, direct or indirect, association to mink had been reported. Cases associated to both mink and direct or indirect contact to other types of livestock was excluded resulting in analysis of mink-only contact cases. Cases with direct mink contact are referred to as primary and cases with indirect exposure (living together with a primary case) are referred to as secondary. Information on each case (age, sex, type of specimen and known livestock contact) were obtained as well. MRSA CC398 isolates from Danish pigs (n=183) described by Sieber et al. (unpublished data) were included in the study for comparison and a total of 88 international isolates described by Price et al. (2012) were included as a reference collection.

**Strain preparation and whole-genome sequencing**

The DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) were used to extract DNA according to the manufacturer’s instructions, including a pretreatment with 5 µg/ml lysostaphin at 37 °C for 30 min, before extraction. The library preparation was performed using Illumina Nextera XT (Illumina Inc., USA) and run on an Illumina NextSeq instrument (Illumina Inc., USA) for 150 bp paired-end sequencing. Whole-genome sequencing data for the international reference collection (Price et al. 2012) were obtained from the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) via BioProject accession number PRJNA274898. The Danish porcine strains were obtained from the Sieber et al. (unpublished data).

**Single nucleotide polymorphism (SNP) calling and phylogenetic analysis**

Using NASP (Sahl et al. 2016), identification of SNP variants was performed using the GATK UnifiedGenotyper with filtering set to remove positions with less than 10 fold coverage and 90% unambiguous variant calls after positions within duplicated regions of the MRSA CC398 S0385 reference chromosome (GenBank accession no. AM990992) was removed using NUCmer. Purging
of the 123 kb recombinant region of the ST398ST9 hybrids was performed prior to phylogenetic
reconstruction using FastTree v2.1.5 as implemented in Geneious v11 (Biomatters Ltd, Auckland,
New Zealand) using the Generalized Time-Reversible model.

Genomic characterization of isolates and clinical characterization of human isolates

The presence of resistance determinants was assessed using raw sequence reads in Mykrobe
Predictor version 0.4.3 (Bradley et al., 2015). Using the same software, the genotype of additional
genes of interest was determined with results filtered for >80% coverage and >5 median depth. The
reference sequences were obtained from NCBI genomes with accession number DQ530361 for
Sa3int, sak, scn and sea, BA000018.3 for sep, KF593809.1 for the cadmium-zinc resistance gene
czc and NC_013450 for genes SAAV_2008 and SAAV_2009 associated with virulence in avian
hosts. Assembled genomes were used to identify SCCmec types, which were determined using
SCCmecFinder (Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/) (Kaya et al.
unpublished data). Furthermore, a subset of assembled genomes, were annotated using Prokka v.
1.2 (Seemann 2014) with subsequent clustering and identification of core and accessory genes using
Roary v. 3.6.0 (Page et al. 2015). Finally, the results were inspected and unique genes were
identified.

RESULTS

Human cases and clinical characteristics

From 2009 till mid-March 2017, a total of 102 human cases reported association to mink, however,
in 22 cases direct or indirect contact to other livestock, such as pigs or cattle, was also listed. Thus,
a total of 80 cases had only mink-only contact registered. The first case of mink-only contact was
recorded in 2011 and the reported numbers increased in the following years from seven in 2011 to
sixteen in 2016 to encompass a total of 80 cases (Figure 1A). Per se, the number of MRSA CC398 cases with mink association (primary and secondary), followed the same increasing trend as the total number of human cases of MRSA CC398 (Figure 1B). Mink-only contacts constituted 80 out of 4,731 MRSA CC398 cases between 2011 -2016.

A larger proportion of the human cases (57%) had infections at the time of isolation dominated by skin and soft tissue infections (SSTI) and with no more serious infections detected such as; respiratory, bone and joint, blood and cerebrospinal fluid. Cases with primary and secondary contact to mink were 67% and 33%, respectively, where 65% of all cases were men and 35% were women. The median age was 35.5 years (range 0-84) and 30.5 years (range 0-61) for men and women, respectively.

**Genomic characterization of isolates**

A total of 162 MRSA isolates were sequenced: 80 from humans, 65 from mink, and 17 from mink feed. A single isolate isolated from mink feed, belonged to CC1 while the rest belonged to CC398. All CC398 isolates carried mecA and had different spa types, with t034 and t011 being most prevalent. Two isolates were identified as the ST398/ST9 hybrid, spa type t899 (Price et al. 2012). The isolates carried SCCmec type IV(2B) subtype a (2/162) and c (1/162), type V(5C2&5)c (145/162) and 14 atypical type V. Most isolates, from all three sources carried crzC (158/162).

Among the human (n=1) and mink (n=2) isolates, three were found to carry Sa3int and scn, while sak was detected in two and three of the isolates respectively. A single human isolate carried sep, while none of the tested isolates were found to carry sea, SAAV_2008 or SAAV_2009.

The resistance profiles of the isolates were determined by detection of specific resistance genes or mutations. The percentage of specific resistance genes detected within the mink and human isolates was largely similar to pig related isolates. All isolates contained mecA, whereas more than 95% of
isolates from all sources contained the resistance genes \textit{tet}K, \textit{tet}M, \textit{bla}Z and \textit{czr}C. The resistance gene \textit{dfr}G was detected in more than 85\% of mink, human and pig isolates. Lower prevalence (\leq 20\%) of \textit{tet}L, \textit{erm}A, \textit{erm}B, \textit{erm}C, \textit{dfr}K, and \textit{aacA}aphD were detected in all groups. Two different point mutations resulting in resistance towards fluoroquinolones were identified at a single amino acid position the \textit{gyr}A gene where mutation \textit{gyr}A\_S84A was the only type found in mink related isolates (10\%), \textit{gyr}A\_S84L was the only type found in human isolates (9\%) and the types found most prevalent in pig isolates (S84L=18\% vs. 2\%=S84A).

\textbf{Phylogenetic analysis}

Isolates from all three sources, human, mink and mink feed, were found to predominantly cluster within the three dominant lineages present in Danish pigs, L1-L3, as shown in Figure 2. Mink feed was represented in all three dominant lineages. In addition, a substantial number of mink-associated isolates, human, mink and feed, (n=20) clustered in a separate clade together with n=3 pig isolates outside lineages L1-L3. The accessory genome of these isolates were analysed and compared to the genomes of the three closest isolates from the reference collection, showing that a total of 33 genes encoding 14 described and 19 hypothetical proteins were present in isolates within the clade, but absent in the genomes of the reference isolates.

The proportions of isolates within each lineage varied. However, the largest proportion of isolates from all sources was found within L3 (Table 1). In single cases, isolates collected at different time points from the same farm belonged to different lineages.

\textbf{DISCUSSION}

We found 1.7\% of all CC398 cases, reported from 2011-2016, to have mink-only contact. No serious infections were observed which point to a healthy worker effect. MRSA CC398 from
human cases with mink contact seems to be associated with the three major MRSA CC398 lineages found to be specific for Danish pigs, hence a spillover of MRSA from the pig reservoir into the mink production result in carriage and infections in people with contact to mink.

An increase in human mink-only cases of MRSA CC398 was observed, elucidating the problematic development of continuous dissemination of MRSA CC398 into new animal reservoirs. At this point a large number (57%) of human mink-only related cases had infections at the time of isolation (Figure 1A). The number of mink-only cases with infections has increased with a similar tendency as seen for total MRSA CC398 cases until saturation of new cases in 2014-2015 (Figure 1B), however this comparison should be interpreted with caution, as it assumes a similar saturation in mink-only contact cases. At this point, only a limited number of mink farms (33%) (Hansen et al. 2017) are positive compared to pig farms (88%) (DANMAP 2016), and it is not known if saturation is reached. The proportion of positive mink farms determined in 2015, 2016 and 2017 has been stable; hence saturation is possible (unpublished data).

Most human cases of MRSA CC398 are found in people with contact to pigs (DANMAP 2012), and this is most likely driven by an increase in positive pig farms (DANMAP 2016), hence the increase seen in people with mink contact is likely to be driven by the increase in positive pig farms and consequently in the mink feed containing raw slaughter products from pigs. The clustering of isolates from all three sources, human, mink and Danish pigs, supported this correlated increase (Figure 2). The age range in both men and women was broad, including cases among infants and children, indicating transmission from farm environment to households as it has been observed in the pig production (Cuny et al. 2009). Some mink farms have other animals on their premises, but we inly included mink-only contact cases in this analysis, and transmission from other livestock, e.g. pigs and cattle, was therefore assumed to be unlikely for human cases in this study. However, a few farms (n=8) from which the mink isolates originated, housed other livestock including pigs,
cattle, poultry or goats which could carry MRSA CC398 and cause within-farm cross-contamination (Crombé et al. 2013). In Denmark, many mink farms are located in high pig-density areas, and local dissemination of MRSA CC398 have been demonstrated (Locatelli et al. 2016). The influence of these other routes of possible transmission is not known in the present study, but it seems unlikely that one third of the Danish mink farms have become contaminated via the environmental route given the possible exposure through the feed.

The dominant spa type t34 followed by t011 belonging to MRSA CC398, in human and mink isolates, were similar to results from the pig production. The resistance patterns seen in strains of MRSA CC398 from all sources were also highly similar. The most suitable genetic marker for pig-associated MRSA CC398 is tetM, according to McCarthy et al. (2012), which was present in >95% of the isolates from mink and humans. Trimethoprim resistance, encoded by dfrG, is normally largely absent in human S. aureus and an indicator of pig-associated MRSA CC398. In the present study dfrG was found in more than 80% of isolates from mink and humans. The additional tetracycline resistance gene, tetK, characteristic for MRSA CC398 SCCmec type (5C2&5) Vc (Li et al. 2011), was present in similar proportion in isolates from pigs, mink and humans. Many different types of antibiotics are used in the mink production, and high prevalence of multiple resistance genes could therefore be expected (DANMAP 2014; Kvist et al. 2017). Interestingly, most mink isolates carried czrC (96%), which confers cadmium-zinc resistance (Cavaco et al. 2010). Zinc is generally not used in the mink production, whereas zinc oxide is highly used in the pig production, approx. 400 tons of zinc a year, to prevent post-weaning diarrhea (DANMAP 2016). The presence of czrC was equally high in human (100%), mink (95%) and pig (98%) isolates. Taken together, the results from analysis of genetic markers strongly indicate MRSA CC398 found in mink and human mink-only cases are similar to the MRSA CC398 found in the pig-production. A suggested genetic marker of human adaption is φSa3 prophage and the IEC related genes (McCarthy et al. 2012).
presence of markers related to human adaption was \(\leq 5\%\) which indicates the isolates found in mink and human mink-only cases to be of animal origin and point to absence of human adaptation. In Denmark, CC1 has also been detected in pigs, however at a lower prevalence compared to CC398, which corresponds to the fact that only a single mink-related isolate belonged to CC1 in the present study.

Clustering of isolates from human mink-only cases and mink (Figure 2), confirmed transmission of the MRSA CC398 found in mink to humans with contact to mink, as observed in other livestock species like pigs and horses (Köck et al. 2013; Islam et al. 2017), where farm workers and veterinarian have been found to carry the same clones as the contact animals. No phylogenetic differences were observed between cases with primary and secondary contact, supporting that no human adaptation has occurred. A recent study by Sieber et al., (unpublished data) showed that three lineages, L1-L3, of MRSA CC398 are now dominating in Danish pig production, and are transmitted to humans with contact to livestock. The proportion of Danish pig isolates located within each clade, showed L3 to be dominant followed by L1 and L2 respectively (Sieber et al. unpublished data), which were similar for the distribution of mink and human isolates found in the present study (Table 1).

A larger proportion of the mink isolates were found within L3 (65\%) compared to isolates from Danish pigs (53\%). The MRSA CC398 isolates from pigs were all from 2014, whereas the mink isolates primarily were from 2015-2016. Sieber et al. (unpublished data) showed a recent rapid increase in prevalence of L3 compared to the other lineages. This could explain the seemingly higher proportion of mink isolates found in L3 compared to pig isolates. The proportion of pig isolates clustering outside L1-L3, were shown to be stable over time (Sieber et al unpublished data). In our study, the proportion of mink and human isolates clustering outside of L1-L3 exceeded that of pig isolates, which is in line with the new clade found to differ from L1-L3, hereafter referred to
as non-L1-L3, marked with a question mark in Figure 2. Within non-L1-L3, isolates originating from all sources include Danish pig (n=3), mink (n=3), mink feed (n=5) and human (n=12). The presence of isolates not clustering with L1-L3 indicates introduction of other lineages which circulate in the Danish pig production, however with less success compared to the three major lineages. The predominance of isolates from human and mink feed within the non-L1-L3 clade, could indicate a buildup due to introduction of another lineage from perhaps; mink feed composed of slaughter products from other animals than pigs (Dietz et al. 2006), mink feed not originating from Denmark and/or from non-Danish workers in mink farms. Humans are known to be an important source of introduction of MRSA CC398 (Grøntvedt et al. 2016). It would be reasonable to assume that other countries have dominant lineages as it is seen in Denmark, where introduction via foreign farm workers or imported slaughter offal used in mink feed could explain the non-L1-L3 clade identified in the present study. Two t899 isolates were found in humans connected to mink in the category of others, which additionally support introduction from other countries or animal sources as t899 is known as a hybrid ST9/ST398 strain (Price et al. 2012). t899 has been isolated from multiple sources (pigs, cattle, poultry and retail food) from European countries including Poland, France, Germany, the Netherlands, Italy and Spain (Larsen et al. 2016; Fetsch et al. 2017). The phylogenetic analysis, where feed isolates were represented in each of the dominating lineages and the presence of strains from the same farm in different clades, point to repeated feed-borne introduction as hypothesized by Hansen et al. (2017).

The confirmation of mink feed as the likely source of introduction, and the lack of mink adaptation, indicate the presence of MRSA CC398 in mink farms to be a contamination rather than an actual persistent colonization. This would imply that mink farms are not an actual persistent reservoir of MRSA CC398, but merely an intermittent reservoir, which may cause transmission to people with contact to mink. This is in contrast to pigs where MRSA CC398 seems to colonize and to be a
permanent reservoir. An investigation of possible colonization of mink and a risk assessment of human colonization from working with farm mink remains to be investigated. In conclusion, this study showed an increasing number of human cases with mink-only contact to be positive for MRSA CC398. Our data supports that these isolates originate from Danish pigs, however transferred to humans via mink feed and mink. Mink was found to be contaminated with the same lineages (L1-L3) of MRSA CC398 as found in the Danish pig production, illustrating a spillover from one production type into another. Further, we show that the transmission route most likely are through contaminated mink feed that continuously introduce MRSA CC398 into mink farms, however human introduction could also a possibility. Actions on breaking the line of introduction should be initiated, as MRSA CC398 in mink constitutes a risk to humans.

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Fig. 1. (A) Annual number of MRSA CC398 from new cases of MRSA-positive persons with contact to mink, Denmark, 2011-2016 (n=74). The category of six cases was unknown and therefore excluded resulting in 74 cases. (B) Development in human cases with infections of all CC398 and CC398 mink-only contact. Cases with mink-only contact are cases with primary or secondary contact to mink and no other type of livestock.
Fig. 2. Phylogenetic relationship MRSA CC398 from multiple sources in Denmark and from a worldwide collection. The phylogeny included 65 isolates from Danish mink from 2014-2016, 16 isolates from mink feed from 2016, 183 isolates related to Danish pigs from 2014 (Sieber et al. unpublished data), 88 isolates included as reference phylogeny from Price et al. (2012). The tree was rooted according to Price et al. (2012). Type of isolate: ref. strains=grey branch, mink=red closed dot, mink feed=red open dot, DK-pig=red branch, human=black closed dot. Clades of the
three major Danish MRSA CC398 lineages are indicated as L1-L3, and an unknown clade of possible interest is marked with a question mark.

Table 1. Diversity of lineages isolated from mink, mink-only human cases and pigs.

<table>
<thead>
<tr>
<th>Source</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>Remainders</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mink</td>
<td>8%</td>
<td>9%</td>
<td>65%</td>
<td>18%</td>
<td>65</td>
</tr>
<tr>
<td>Human mink-only cases</td>
<td>20%</td>
<td>3%</td>
<td>36%</td>
<td>30%</td>
<td>80</td>
</tr>
<tr>
<td>Danish pigs*</td>
<td>21%</td>
<td>16%</td>
<td>52%</td>
<td>10%</td>
<td>183</td>
</tr>
</tbody>
</table>

* Sieber et al. (unpublished data)
4. General discussion and concluding remarks

The following section will provide an additional discussion regarding results and experiences obtained, during the four studies presented in the present PhD project. Some issues already discussed in the manuscripts and new information, obtained as part of but not included in the manuscripts, will to some extent be discussed when relevant. The purpose of this section is to sum up the knowledge achieved as part of the PhD project, and highlight the contributions hereof in relation to the general issues stated as part of the introduction.

Prior to the results obtained and presented in manuscript 1, little information existed on the within farm levels of MRSA CC398 in pig farms. The study presented in manuscript 1 showed that it is possible to quantify MRSA CC398 from a contaminated farm environment, by direct plating of animal swab samples or plate counts of air samples collected by active air sampling. The selective and indicative properties of Brilliance MRSA2 agar were found sufficient to distinguish between MRSA CC398 and additional flora. A small pilot study, prior to study 1, where MRSA selective plates from bioMérieux, chromID® MRSA, were tested, found the specificity of those plates to be insufficient in order to distinguish MRSA from additional flora. One organism in particular, Rothia nasimurium, tended to have similar morphology as MRSA CC398 on these plates. As highlighted in the sub-section “Quantification of MRSA CC398” page 31, there are multiple challenges regarding quantification of LA-MRSA with molecular methods and we therefore chose to quantify MRSA CC398 by plate counts. The limitation of not being able to obtain standardized animal samples from swabbing still applies. However the method assessment carried out in study 1, found a larger proportion of variance explained by pig compared to biological replicate, cooperating the methodological limitation to be less important. Yet, an extended verification of the robustness of the swabbing method could have been carried out by including more pigs.

The highest levels of MRSA CC398 were found within the weaning unit, which was to be expected, due to the selective pressure applied through extensive usage of tetracycline and zinc oxide in this unit (Moodley et al., 2011). This finding is worrisome, because the pig-to-human contact is intensive in the weaning-unit which increases the farmer’s risk of becoming carrier and potentially act as a vector of
MRSA transmission from the farm environment into the community (Graveland et al., 2011b). Knowledge from the current study therefore provides evidence of the weaning unit as an optimal unit for implementation of intervention initiatives. Importantly, when assessing the effect of interventions, samples from both the animals and the environment should be included to substantiate the effect of interventions, as we find the correlation between the two sample types to be insufficient to recommend only using air or animal samples.

Study 1 thus provided new knowledge regarding the within farm levels of MRSA CC398 and the high levels detected in the weaning unit support the concern of spillover into humans and possible unknown animal reservoirs too. This possible spillover to other animal reservoirs was the hypothesis underlying the study described in manuscript 2. Knowledge regarding high prevalence of LA-MRSA in veal calves (Graveland et al., 2010) and to some extent dairy herds in the Netherlands (Tavakol et al., 2012), a country with similar prevalence of positive pig herds as the Danish prevalence, supports the initiation of the screening of MRSA CC398 in Danish veal and dairy herds as described in manuscript 2. A spillover from the pig production into both production types was shown from the phylogenetic analysis. The screening of veal calves found only 2/17 of the farms and 4/620 of the animals to be MRSA CC398 positive, where we were unable to detect MRSA from the animals at a follow-up sampling. These observations strongly suggest that veal farms do not represent a new significant reservoir of MRSA CC398, but we should be aware that introductions do occur. Evidence of the ability of MRSA CC398 to adapt to and survive in cattle, has been provided from multiple countries but in Denmark at this point, it seems as we can prevent the emergence of veal calves as a reservoir (Bos et al., 2012). Danish dairy herds were found to represent a low prevalence reservoir of MRSA CC398, and the detection of the first clinical case of mastitis caused by MRSA CC398 highlights the importance of limiting the expansion of the current low prevalence reservoir. In isolates from BTM, we found indications of possible adaption to a non-pig niche, based on the loss of czrC, which is interesting, however worrisome. Currently, we are investigating if the BTM isolates, from study 2, carry bovine associated virulence genes which would provide evidence of adaption to cattle. At this point, it might be possible to arrest the development of MRSA CC398 as a permanent part of the dairy production, something which has been found highly difficult once high prevalence is established, as seen in pigs (Miljø-og Fødevareministeriet, 2017). The first Danish isolate of mecC-MRSA in dairy herds was
found, something which should be monitored as mecC-MRSA previously has been shown to be associated to bovines (García-Álvarez et al., 2011; Petersen et al., 2013) and therefore might be prone to flourish within this production line. Altogether, study 2 thus provides important knowledge on the MRSA CC398 status of Danish cattle, knowledge that might aid the dairy and meat industry to handle MRSA CC398 before the prevalence rises.

Spillover from Danish pigs into previously unidentified reservoirs was illustrated in study 2. A similar dissemination into Danish horses has been reported (Islam et al., 2017) and cooperate the need for screening of possible unknown reservoirs. The Danish mink production has gained our attention for some time, as cases of people with mink contact have been found MRSA CC398 positive since 2009. However not until 2013 was MRSA CC398 identified in mink, at the National Veterinary Institute. The increased awareness of MRSA CC398 positive mink led to a screening of clinical submissions of mink upon arrival to the National Veterinary Institute, which was followed up by an on farm screening in 2015. Surprisingly, a prevalence of around 33% of farms was detected and as part of study 3, the Danish mink production was substantiated as a new reservoir of MRSA CC398. The type of MRSA found within mink farms and positive feed samples pointed to pigs as the source of introduction, possibly via contaminated mink feed. The large number of people, potentially exposed to MRSA CC398 through employment in the Danish mink production encouraged the initiation of study 4. The detection of the source and degree of human infections caused by mink contact is important to assess, if we want to try to reverse the spread of MRSA within the mink industry. From a screening of the human cases reported to Statens Serum Institute, the number of human cases with contact to mink as the only type of livestock had increased since 2011, emphasizing the importance of monitoring this reservoir. A phylogenetic analysis and genomic characterization of isolates originating from Danish pigs, humans with mink-only contact, mink, and mink feed, showed a spillover from pigs into the mink production from where the humans sustained asymptomatic carriage or infections. Feed was identified as the most likely source of introduction.

A geographical perspective was not applied in study 4, investigating the location of mink farms compared to MRSA positive pig herds. This could be interesting to investigate as spillover into the general community and nearby animal production sites, in areas with high density of LA-MRSA positive pig herds is possible and could be another explanation for the emergence of MRSA CC398 in
mink (Locatelli et al., 2016). However, it seems highly unlikely that one third of the Danish mink farms have become contaminated via the environmental route. The detection of MRSA CC398 in mink feed of the same lineages as found in pigs and mink, cooperate transmission via feed-borne contamination as the most likely route of introduction. However, another question which could be discussed further is whether mink in fact is an actual reservoir of MRSA CC398. We have shown a high prevalence of positive mink farms, however we do not know if the animals are colonized the same way as pigs are colonized or merely contaminated by their feed. A within herd screening is currently being carried out and preliminary results show an animal prevalence ranging from 20 to 29% (Fertner et al., 2017). Additionally, a study assessing how long mink stay MRSA CC398 positive, if removed from the contaminated environment and contaminated feed is currently being carried out, and preliminary results indeed suggest that MRSA CC398 is lost within a short period of time (Dr. Fertner, personal communication). Nonetheless, mink represents a reservoir of human exposure, even if they are not truly colonized, and the presence of MRSA CC398 primarily on the paws and in the pharynx, poses a human health hazard to farmers, as they risk getting bites and scratches from infected sites, when handling the animals and. Taken together, study 3 and 4 provides important knowledge on the MRSA CC398 status of Danish mink farms and highlights the likely transmission route via feed. This enables the industry to have focus on the human health hazard to farmers and to initiate interventions to arrest the continuous introduction, hopefully resulting in elimination of this newly identified reservoir.
5. Achievements of the PhD project and perspectives

One of the overall achievements of the present PhD project is the establishment of low-cost quantitating methods for MRSA CC398 in animals and farm environments. Currently these methods are used to assess different intervention strategies applied in pigs. At present, the cost of applying the Norwegian approach to eradicate LA-MRSA in the pig production in Denmark, has been found to be so high, 14-15 billions dkk. (Olsen et al., 2017), that it seriously questions the feasibility of applying this strategy if we want to continue the Danish production of conventional pigs. Therefore, the future focus should be on keeping MRSA CC398 within pig farms, eliminate the dissemination to other productions, and reduce the within herd level, perhaps to a degree where only limited contamination of farm workers is observed. Regarding expansion of CC398 in Denmark, the present PhD study verified that spillovers into other animal reservoirs indeed occur, including spillover to cattle and mink, and that the mink production unexpectedly is positive of MRSA CC398 in one third of the tested farms. Study 4 highlighted the risk of exposure to a large number of people, but simultaneously provided knowledge regarding the source of introduction enabling initiation of interventions. Future screenings should also include the Danish poultry production as very limited knowledge exists regarding MRSA CC398 in Danish poultry (Miljø-og Fødevareministeriet, 2017). The results obtained regarding spillover emphasize the need for continued screening of low prevalence MRSA CC398 positive productions and possibly unknown positive productions, to try to take control of the development, emergence and spread of MRSA CC398 in Denmark. This knowledge applies to other countries with current national low prevalence of human MRSA cases too. Conclusively, the comprehensive work included in this thesis illustrated the crucial importance of identifying potential new LA-MRSA reservoirs with possible relevance to human health.
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