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# Importance of Methicillin-Resistant *Staphylococcus aureus* in swine in Denmark

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# Table of content

## List of abbreviation

BSI - Bloodstream infection

CA-MRSA - Community-associated MRSA

CC398 - Clonal complex 398

DVFA - Danish Veterinary and Food Administration

Fab - Fragment antigen-binding

FC - Fragment crystallizable

FnBP - Fibronectin-binding protein

HA-MRSA - Healthcare-associated MRSA

IgG - Immunoglobulin G

LA-MRSA - Livestock-associated MRSA

MHC - Major histocompatibility complex

MLST - Multilocus sequence typing

MRSA - Methicillin-resistant *Staphylococcus aureus*

MSSA - Methicillin sensitive *Staphylococcus aureus*

PBP2a - Penicillin binding protein 2a

PFGE - Pulsed-field gel electrophoresis

PMS - Phenol soluble modulin

PVL - Panton-Valentine leukocidin

SAg - T cell superantigen

*S. aureus* - *Staphylococcus aureus*

SCCmec - Staphylococcal Cassette Chromosome

SE - Staphylococcal enterotoxin

SE-l - Staphylococcal enterotoxin-like superantigen

SpA - Staphylococcal protein A

SSTI - Skin and soft tissue infection

TSST-1 - Toxic shoch syndrome toxin-1

WGST - Whole-genome sequence typing

# 1. Abstract

In Europe, LA-MRSA CC398 is spreading in pig farms. The results in my thesis demonstrate that a possible explanation of the fast spread within pig herds can be due to movement of pigs through the production pyramid and indirect introduction. Pigs serves as a reservoir for LA-MRSA CC398, and this reservoir may pose a risk for human either being colonized or infected by this pathogen, especially for those working in the pig sector, such as farmers or veterinarians, but also for the general community.

During the recent years, the prevalence of LA-MRSA CC398 has grown fast from 0% from the first screening to above 80% in later screenings in the Danish conventional pig herds. In parallel, human testing positive for LA-MRSA CC398 with or without livestock contact has increased in this country.

Despite the action plan Denmark implemented, the LA-MRSA CC398 is still increasing in pig herds in this country, and due to its ability to transmit from animals to humans, it may pose a threat to the public health.

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## 2. Introduction

My first encounter with methicillin-resistant *Staphylococcus aureus* (MRSA) was when I, before starting my studies in Budapest, worked at a nursing home in Norway. One of the patients at the nursing home got infected by MRSA and the infection was hard to cure. After this I have been curios about MRSA and wanted to learn more about the bacterium.

*Staphylococcus aureus (S. aureus)* can be the cause of a wide range of infections. There are several antibiotics available to treat infected individuals. On the other hand, antibiotic resistance is on the rise, and treatment failures are associated with high human and medical costs (Vestergaard et al., 2019). *S. aureus*, particularly MRSA, has evolved its survival techniques in response to high antibiotic selective pressure and has quickly transformed into a multidrug-resistant organism (Hong et al., 2016).

The rise of multidrug-resistant virulent MRSA strains has become a significant public health issue. MRSA is a worrying human pathogen and a historically emerging zoonotic pathogen of public health and veterinary concern (Algammal et al., 2020). For a long time, MRSA has been recognized as a significant cause of healthcare associated infections in humans. With time, strains of MRSA have also been detected in community-associated infections and, more recently, strains have been detected in livestock and companion animals (Sergelidis and Angelidis, 2017).

Based on its origin, MRSA has been divided into different groups: healthcare-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) (Price et al., 2012). I will in this thesis review research on the subject as a whole, and also look more closely on how LA-MRSA has spread in the Danish pig industry and its importance in Denmark. As LA-MRSA clonal complex 398 (CC398) has expanded across pig herds and farms of Europe during the past decade. The bacterium is now a significant source of human colonization and infections in nations with historically low MRSA levels, such as Denmark, which has seen a spike in MRSA levels in recent years (Sieber et al., 2018).

# 3. Literature review

## 3.1 *Staphylococcus aureus* (General characteristics)

Alexander Ogston first discovered *S. aureus* in 1880 when he identified it from a surgical wound infection (Lakhundi and Zhang, 2018). The bacterium is a Gram-positive coccus belonging to the family of *Micrococcacea*. The cells can appear individually or in pairs. If dividing cells do not split, they can form "grape" like formations, unique for these bacteria (Stapleton and Taylor, 2002). Pigment production can be observed on colonies inoculated in blood agar, having a characteristic gold or white appearance. Growth can occur both in aerobic and anaerobic conditions, and most strains can ferment mannitol anaerobically. *S. aureus* can produce catalase and coagulase (Brown et al., 2005). It is divided into two subspecies, *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* (Götz et al., 2006).

*S. aureus* can colonize and cause infection in many different hosts including humans, livestock, companion animals, and wild animals (Espinosa-Gongora et al., 2014; Matuszewska et al., 2020). When a host is colonized, it may act as a reservoir of infections in other host species (Matuszewska et al., 2020). With this wide range of different hosts and infections, it poses a serious threat to animal health, food security, and, as a result, human and public health (Peton and le Loir, 2014). In the veterinary aspect, *S. aureus* is considered one of the most crucial pathogenic staphylococcal species, with a significant impact on both animal health and welfare and the economic situation for the livestock industry (Peton and le Loir, 2014).

*S. aureus* can be discovered frequently on human and animal skin and mucosa, and about half of the human population are colonized with it, usually in the nasal or oral mucosa. The hosts can be persistent, intermediate, or non-carriers, and it can cause various diseases as it is a facultative pathogen (Crespo-Piazuelo and Lawlor, 2021; DANMAP, 2019). Its ability to cause a wide range of infections makes it a very successful pathogen. The infections vary, among other things, from skin infections, like abscesses, to bacteriaemia causing infections, like endocarditis, osteomyelitis, septic arthritis, and epidural abscesses. This wide variety of infections can be explained by the extensive virulence factors of this bacterium (Archer,

1998). Despite of this, *S. aureus* is not a frequent cause of severe diseases in healthy individuals (Liu, 2009).

### 3.1.1 Diseases in different hosts

There is extensive research on how *S. aureus* affect animals. These bacteria can cause different diseases in different animals.

*S. aureus* is considered one of the biggest causes of mastitis in livestock and has a significant impact on the milk production industry (Peton and le Loir, 2014). It is known to cause subclinical and clinical mastitis, characterized by an increase in the somatic cell count. Mastitis caused by *S. aureus* is a significant issue in the industry, concerning animal health and financial loss due to a reduced milk yield and quality. Transmission of these bacteria between cows typically occurs during milking, as the infected quarter appears to be the primary reservoir of *S. aureus* (Monistero et al., 2018).

In horses, *S. aureus* infections can cause abscesses and cellulitis in the skin. Botryomycosis, a pyogranulomatous inflammation of the udder, can be seen in the mare, cow and sow after infection with this bacterium (Peton and le Loir, 2014).

*S. aureus* is one of the most common causes of bacterial infections, causing arthritis, abscesses, gangrenous dermatitis, and septicemia, among other diseases in poultry (Haag et al., 2019). Infection by *S. aureus* can result from open wounds or occur during hatching as a result of contamination caused by an open umbilicus (Peton and le Loir, 2014). Another disease commonly seen in poultry is bumblefoot, also known as ulcerative pododermatitis, a significant animal welfare issue that may result in economic losses. *S. aureus* is one of the most commonly cultured pathogens from this disease, although other factors such as management, nutritional factors, density and litter factors also play a crucial role (Olsen et al., 2018).

In rabbits, *S aureus* causes infections such as suppurative dermatitis, abscesses, pododermatitis, and mastitis. These infections in rabbit farming cause significant economic losses (Haag et al., 2019).

*S. aureus* subsp. *anaerobius* is the causative agent of abscess or Morel's disease in sheep and goat, it affects young individuals and is a non-fatal and contagious disease characterized by abscess formation in or adjacent to the superficial lymph nodes (Musa et al., 2012).

Pigs are frequently carriers of *S. aureus* but rarely show clinical signs. However skin infections caused by *S. aureus* can be observed in pigs. *Staphylococcus hyicus* is the staphylococcal species more often responsible for skin infections (Verkade and Kluytmans, 2014; Fluit, 2012). Botryomycosis can also occur after *S. aureus* infection as mentioned above (Peton and le Loir, 2014).

## 3.2 Virulence factors

Virulence factors include capsular polysaccharides, surface-associated proteins, extracellular toxins, and extracellular enzymes (Algammal et al., 2020). These factors enable *S. aureus* to attach to the surface, invade or escape the immune system and inflict harmful toxic effects on the host (Bien et al., 2011).

### 3.2.1 Capsular polysaccharide

Certain *S. aureus* strains are protected by a polysaccharide capsule (microcapsule) (Chavakis et al., 2007). The polysaccharide capsule enhances the virulence of the bacterium by inhibiting complement and antibody-mediated opsonization and phagocytosis (Algammal et al., 2020). The capsule successfully prevents phagocytic cells from recognizing the bacterial surface and surface-associated proteins, such as opsonins (Kuipers et al., 2016).

### 3.2.2 Surface associated proteins

The most typical surface-associated protein of *S. aureus* is Protein A (SpA) (Foster and McDevitt, 1994). It can bind to circulating immunoglobulin G (IgG), inhibit opsonization by the complement system, and shield the bacterium from phagocytosis (Algammal et al., 2020). SpA bind to IgG on the fragment crystallizable (Fc) region, and the outcome of the binding is a coated bacterial cell wall with IgG positioned improperly, preventing recognition by neutrophil receptors (Foster et al., 2014). Along with its Fc-binding capacity, SpA can bind to the fragment antigen-binding (Fab) region of the beta-cell receptor, acting as a beta-cell superantigen, inducing programmed cell death (Kobayashi and DeLeo, 2013). Another surface-associated protein of *S. aureus* is the fibronectin-binding protein (FnBP). FnBP binds to fibronectin of the host cell and links the bacterium and host cell, initiating uptake into the host cell by endocytosis (Foster, 2016). Uptake can also occur by cells that are usually not phagocytic, such as endothelial and epithelial cells, which can facilitate the spread of bacteria from the bloodstream to internal organs (Burke et al., 2010).

### 3.2.3 Extracellular toxins

The secreted toxins of *S. aureus* have an essential part in its virulence (Oliveira et al., 2018). They can be parted into cytotoxins, cytotoxic enzymes, and superantigens (Tam and Torres, 2019).

Cytotoxins attack the membranes of host cells (Tam and Torres, 2019). These toxins have the ability to lyse the cells by forming beta-barrel pores (Bien et al., 2011), and allowing essential molecules and metabolites to escape (Otto, 2014). This can lead to death of the target cell (Vandenesch et al., 2012). Several cytolytic toxins are produced by *S. aureus*, and perhaps the most well-known of them is alpha-toxin. In addition, *S. aureus* produces several bi-component toxins belonging to beta-barrel pore-forming leukocidins, with structural similarities to alpha-toxin (Otto, 2014). Panton-Valentine leucocidin (PVL), among others, are a leukocidin related to infection in humans (Tam and Torres, 2019).

Phenol soluble modulins (PMS) are peptides facilitating the lysis of cells in order to evade immune cell clearance. PSM is thought to attach non-specifically to the cytoplasmic membrane disintegrating the membrane, in addition, oligomers can aggregate and form a

short-lived pore as seen on figure 1 below (Oliveira et al., 2018). PMS plays several roles in the pathogenesis, including lysing of red and white blood cells, inducing inflammatory responses, and aiding in the formation and spread of biofilm-associated infections (Peschel and Otto, 2013).

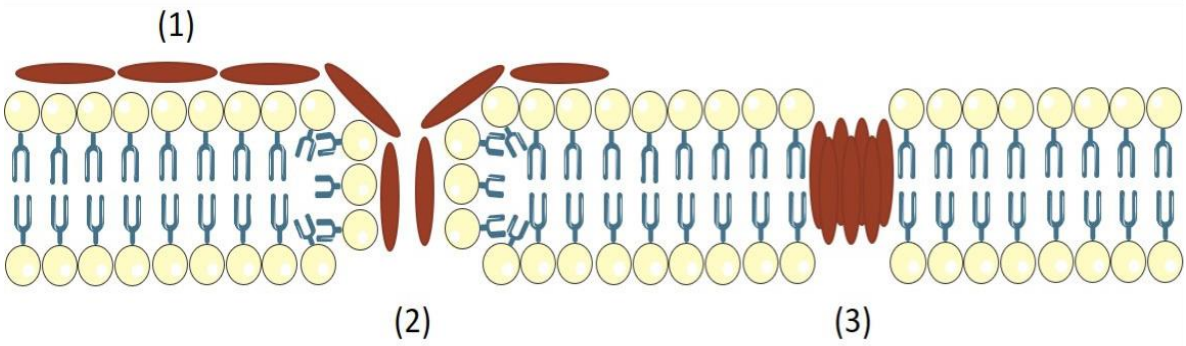


Figure 1. Showing the attachment, disintegration and pore formation of PSM in the cytoplasmic membrane (Oliveira et al., 2018).

Beta-toxin is a cytotoxin produced by *S. aureus*. It is a sphingomyelinase that converts sphingomyelin into ceramide and phosphocholine (Tam and Torres, 2019). Beta-toxin has the ability to lyse red blood cells, allowing the bacterium to bypass the host immune system and acquire nutrients (Huseby et al., 2007). Beta-toxin has a species-dependent hemolytic activity that is proportional to the quantity of sphingomyelin in the red blood cells. Sheep, goats and cows are extremely sensitive, humans and rabbits are less sensitive and canine red blood cells are resistant (Tam and Torres, 2019).

T-cell superantigen (Sag) are the most abundant type of exotoxin produced by *S. aureus*. They can be split into three broad categories: staphylococcal enterotoxin (SE), staphylococcal enterotoxin-like (SE-l) superantigens, and toxic shock syndrome toxin-1 (TSST-1) (Tam and Torres, 2019). These exotoxins all work by activating massive numbers of T-lymphocytes (Xu and McCormick, 2012). They do this by binding to the major histocompatibility complex (MHC) class II molecules (on antigen-presenting cells) and the variable region of T-cells as seen on figure 2, causing the T-cells to produce vast quantities of pro-inflammatory cytokines. This can result in fever, rash diarrhea, hypotension, and multiple organ failure. Following the event, T-cells might fail to proliferate or secrete cytokines, or cell death can occur (Oliveira et al., 2018).

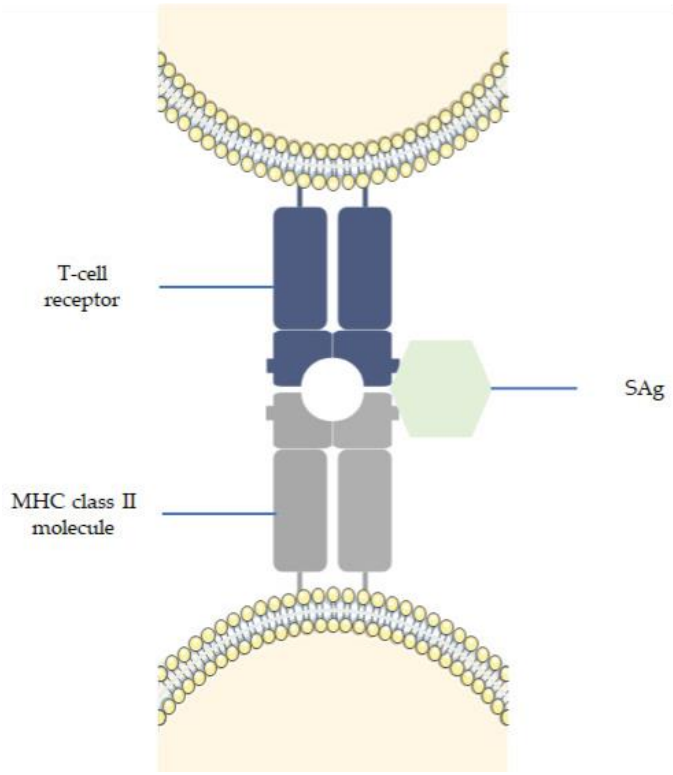


Figure 2. The binding of Sag to the MHC class II and T-cell receptor (Oliveira et al., 2018).

### 3.2.4 Extracellular enzymes

Extracellular enzymes produced by *S. aureus* can be coagulase, von Willebrand factor binding protein, and staphylokinase. These cofactors do not have an enzymatic effect on their own, but they can activate host enzymes. They can take over some aspects of the coagulation system of the host; thus, the innate immune defenses of the host are manipulated in order to promote bacterial survival and dissemination (Tam and Torres, 2019). In addition, *S. aureus* produces a broad range of additional exoenzymes, including nucleases, proteases, lipases, hyaluronidase, and collagenase capable of generating bacterial nutrients through host tissue breakdown promoting bacterial growth and increasing the potential for invasive disease (Berends et al., 2010).

## *3.3* Penicillin resistance of *S. aureus*

### 3.3.1 History

Prior to the antibiotic era, individuals infected with pathogenic *S. aureus* had a mortality rate of more than 80% (Fuda et al., 2005). Alexander Fleming discovered in 1928 a zone surrounding an invading mould on an agar plate where bacterial growth was inhibited, and later this led to the discovery of penicillin (Gaynes, 2017). Mass production of penicillin started after discovering its bactericidal effect (Lakhundi and Zhang, 2018). During World War II, penicillin proved to have good efficiency in treating bacterial infections of soldiers, but unfortunately, it did not take a long before strains resistant to penicillin started to cause concern (Ventola, 2015; Stapleton and Taylor, 2002). Two years after penicillin was introduced, the first penicillin-resistant strain of *S. aureus* was discovered (Lakhundi and Zhang, 2018).

### 3.3.2 Structure

Penicillin belongs to beta-lactam antibiotics, which are characterized by being in possession of the four-membered beta-lactam ring necessary for their biological activity. Penicillin acts by inhibiting the cross-linking of peptidoglycan, which is the most essential component of the bacterial cell wall. This will result in the lysis of the bacterial cell (Vestergaard et al., 2019).

### 3.3.3 Beta-lactam

As mentioned, the bacterial cell wall is made up of peptidoglycans, composed of glycan chains consisting of N-acetylglucosamine and N-acetylmuramic acid that are cross-linked by short stem peptides. Polymerization of the glycan strand and cross-linking of the short stem peptides between the glycan chains is mediated by penicillin-binding proteins (Sauvage et al., 2008). Beta-lactam antibiotics work by binding to penicillin-binding proteins of the bacterial cell wall where they serve as suicide substrates, and by that, prevent the bacteria from synthesizing the cell wall (Zervosen et al., 2012). In this way, the production of the peptidoglycan layer is disrupted, and cell wall lysis can occur (Deurenberg et al., 2007).

### 3.3.4 Beta-lactamase

*S. aureus* strains displaying resistance for penicillin can produce an extracellular enzyme called beta-lactamase, which manages to inhibit the activity of penicillin (Stapleton and Taylor, 2002). Beta-lactamase hydrolyzes the beta-lactam ring of penicillin, leaving it inactive. Beta-lactamase is encoded by the *blaZ* gene, and this gene can be included on mobile elements such as transposons, which can either be incorporated into the chromosome or found on plasmids, which frequently can contain further genes resistant to other antibiotics and heavy metals (Vestergaard et al., 2019).

## *3.4* Methicillin resistance of *S. aureus*

### 3.4.1 History

Following the discovery of penicillin-resistant strains, the development of new beta-lactam antibiotics started (Ventola, 2015). Methicillin was introduced in 1959 with the aim of fighting penicillin resistance (Stryjewski and Corey, 2014). The goal was to create a penicillin derivative that could withstand lactamase hydrolysis, but unfortunately, methicillin-resistant *S. aureus* strains were discovered almost instantly when the clinical usage of methicillin was initiated. However, beta-lactamase was not the reason behind methicillin resistance, but rather the development of an additional penicillin-binding protein, termed penicillin binding protein 2a (PBP2a) (Stapleton and Taylor, 2002).

### 3.4.2 Structure

Methicillin is similar to penicillin in structure, besides the benzylpenicillin phenol groups are substituted by methoxy groups. The methoxy groups create steric hindrance around the amide bond, which reduces the amide bonds attraction to staphylococcal-lactamases (Stapleton and Taylor, 2002).

### 3.4.3 Penicillin-binding protein 2a

*S. aureus* strains displaying resistance to methicillin harbors the *mecA* gene. The *mecA* gene codes for the PBP2a, which has a lower affinity to methicillin, and in respect of this, the peptidoglycan layer and cell wall formation can proceed (Deurenberg et al., 2007). The gene is present on the Staphylococcal Cassette Chromosome mec (SCCmec), which is a mobile genetic element (Graveland et al., 2011). The SCCmec are separated into different types ranging from I to XII, and they differ in size. For instance, LA-MRSA has different SCCmec cassettes compared to HA-, and CA-MRSA, namely SCCmec type IVa or V (Lakhundi and Zhang, 2018). The different SCCmec elements contains varied amounts of incorporated plasmids and transposons, which frequently include further antibiotic resistance genes. This can result in multi-resistant phenotypes, which limits treatment options (Ray et al., 2016).

## *3.5* Methicillin-resistant *Staphylococcus aureus*

MRSA is a highly pathogenic and zoonotic biovar of *S. aureus* that meets particular requirements for methicillin resistance as mentioned above (Algammal et al., 2020). The first observations of MRSA were from hospitalized patients in the 1960s, and infections were restricted to hospitals, affecting mostly older or very young individuals, immunocompromised patients, or patients having surgery. In the 1990s, the epidemiology of MRSA infections evolved when the infection rate without the risk factors associated with obtaining HA-MRSA grew rapidly (Gajdács, 2019). Outbreaks of MRSA were seen in healthy individuals, and risk factors were associated with human-to-human interactions in situations such as sports, school, day-care facilities, army, and prisons. The term community-associated MRSA was used to refer to these cases. When looking at the genetic background of CA-MRSA strains, they have distinct sequence types and SCCmec types. Additionally, PVL is commonly seen in CA-MRSA strains (Graveland et al., 2011). In recent years, MRSA has become a regular colonizer of animal populations, presumably aided by the widespread use of antibiotics in this industry assisting the growth of these resistant bacteria by inhibiting the growth of others. The zoonotic MRSA strain has been identified in livestock, and especially in pigs, and has been named livestock-associated MRSA (Pantosti, 2012).

While MRSA infection is found worldwide, there is no particular pandemic strain. Rather than that, MRSA is more likely to arise in waves, which are frequently characterized by the recurrent emergence of dominant strains (Turner et al., 2019). The prevalence of MRSA varies significantly across Europe. In Northern Europe, the prevalence is relatively low compared to other areas of the continent. This can be a result of the strict MRSA infection control strategies. In Southern Europe, the prevalence is higher, particularly high in hospital settings, having up to 50% prevalence of invasive isolates (Graveland et al., 2011). This can be explained partially by the significant variations in screening, isolation, and treatment of patients and hospital staff in different nations. For example, the "search and destroy" policy has been used in the Netherlands and the Scandinavian countries. There is also a low prevalence of MRSA in these countries due to the limited use of antimicrobials in humans (Graveland et al., 2011). In respect of the global health, the increasing prevalence of MRSA in the healthcare section, community and livestock is worrisome due to the fact that these strains show resistance to a wide spectrum of beta-lactams with clinical importance, including penicillin, the majority of cephalosporins and carbapenems (Islam et al., 2020)

## 3.6 Livestock associated MRSA

Even though MRSA has been documented in animals for a long time, a novel linage called CC398 appeared in livestock recently, having a zoonotic potential (Price et al., 2012). LA-MRSA CC398 was first discovered in the Netherlands in 2003 (Verkade and Kluytmans, 2014). Since then, this lineage has emerged as a major source of human infections, most frequently connected to those with animal contact (Price et al., 2012). Humans infected with LA-MRSA was first identified in the early 2000s among swine farmers in France and the Netherlands (Fessler et al., 2018). Two studies from these countries demonstrated the first reservoir of MRSA in animals having zoonotic potential. The isolates belonging to CC398, which, at the time, was relatively rare among humans (Larsen et al., 2015).

In general, *S. aureus* and MRSA have the capability of infecting people, and LA-MRSA CC398 is no exception. It is known to enter hospitals causing nosocomial infections, including postoperative surgical site infections, ventilator-associated pneumonia, septicemia and infections following joint replacement (Cuny et al., 2015). It raises public health

concerns, and especially in nations with intense livestock production, where livestock can function as a potential reservoir for MRSA colonization and infection. LA-MRSA CC398 has so far been isolated from domesticated animals such as cattle, horses, chicken, and turkey, but the pig is presumably its primary host. In livestock, LA-MRSA is seldomly the cause of infections, and pigs are often colonized asymptomatically (Cuny et al., 2015)**.**
While other strains of LA-MRSA have been identified, CC398 dominates in the European livestock (Larsen et al., 2015). The most frequently associated spa types within CC398 are spa t001, t034, and t108 (Ballhausen et al., 2017). In other places of the world, Asia for instance, clonal complexes such as CC9 are more dominant **(**Goerge et al., 2017)**.**

LA-MRSA can be further characterized by not being typable by pulse field gel electrophoresis (PFGE) using the enzyme SmaI, and the majority of this group is resistant to tetracyclines (Li et al., 2011).

In the period from 1970 to 2000, MRSA was not frequently isolated from animals, and if it was, it was presumed to be originating from humans, proved by bio-typing. The animal reservoir was believed to have little value since they had minimal relevance for MRSA-related infections of humans until the end of the 20$^{th}$ century (Graveland et al., 2011). Epidemiological investigations revealed that LA-MRSA CC398 was successfully crossing the species barrier, colonizing and infecting humans (Van Alen et al., 2017). Human infections have become more prevalent in recent years and have also been introduced to human health care systems (Ballhausen et al., 2017).

LA-MRSA CC398 can be transmitted via livestock to humans, especially those working with animals such as farmers and veterinarians, as direct contact with livestock is regarded as the primary risk factor for zoonotic transmission of LA-MRSA CC398 (Van Alen et al., 2017; Ballhausen et al., 2017). Countries with intense industrial pig production, for example, Denmark, have experienced a rise in human infection rates caused by the zoonotic LA-MRSA CC398 (Larsen et al., 2017). Following the first report of LA-MRSA CC398 colonizing conventionally raised pigs, several reports from countries with prominent conventional pig farming, such as the Netherlands, Denmark, Germany, France, and Italy, as well as later reports from North America, Northern Africa, Asia, and Australia, were published (Cuny et al., 2015)**.**

### 3.6.1 Typing methods

MRSA CC398 differs genetically from other MRSA strains by being non-typeable with pulsed-field gel electrophoresis (PFGE) using the restriction enzyme SmaI. This and other factors like the numerous spa types has made genotyping difficult. Luckily this has been aided by whole-genome sequence typing (WGST) (Larsen et al., 2015). To trace the origin and conduct evolutionary studies, whole-genome sequencing can be used as it generates a better genetic fingerprint compared to the conventional methods including multilocus sequence typing (MLST) and spa sequence typing. MLST characterizes LA-MRSA CC398 and is helpful for comparing it to other *S. aureus* clonal complexes, but for assessing group variations, it is ineffective. The disadvantage with spa sequence typing is the small number of related spa types among the LA-MRSA CC398 isolates (Price et al., 2012).

### 3.6.2 Host adaptation

The evolution of the LA-MRSA CC398 lineage is significant for MRSA epidemiology and worldwide health, by virtue of its quick rise and human impact (Price et al., 2012).
After the emergence of LA-MRSA CC398, its origin has been debated. According to research the host adaptability may be dependent on the loss and/or acquisition of mobile genetic components. When LA-MRSA CC398 was compared to other *S. aureus* lineages, it was discovered that the majority of genetic alterations occurred within the accessory genome segments (Ballhausen et al., 2017).

A study on host adaptation implements that assumingly, based on the whole-genome sequence typing phylogeny, LA-MRSA CC398 originates from methicillin-susceptible *S. aureus* (MSSA) associated with humans. On the WGST-based phylogenetic tree, the isolates that made the most basal clades were almost all human MRSA strains. Based on this, it may look like LA-MRSA CC398 has been reintroduced into its original host, namely humans (Price et al., 2012).

It seems that there are two epidemiologically and distinct groups of LA-MRSA CC398 circulating. One group is the human-adapted subpopulation, and the other group is the livestock-adapted subpopulation believed to have been derived from the human subpopulation (Larsen et al., 2016). The reintroduction of LA-MRSA CC398 to animals

from humans was followed by a loss of the phage-carried human virulence genes, as seen on figure 3 below (Price et al., 2012). The beta-hemolysin-converting phage encodes for proteins, which shields *S. aureus* from the innate immune response of humans (Larsen et al., 2016). The immunomodulatory genes which are contained within beta-hemolysin-converting phage are important for human niche adaptation (Price et al., 2012). The group primarily seen in livestock is named CC398-IIa, and the many other basal lineages seen in humans are named CC398-I/II-GOI (Larsen et al., 2015). The difference between the lineages is that CC398-IIa is commonly positive for tetracycline-resistant gene *tet(M)* and negative for staphylococcal inhibitor gene *scn*, and genes coding for PVL are absent, whereas, for CC398-I/II-GOI, it is the opposite (Larsen et al., 2015). After the introduction of LA-MRSA CC398 in livestock, it is suggested that it acquired the SCCmec element and methicillin resistance. According to epidemiological data, it is suggested that LA-CC398 might have reduced virulence and lesser transfer rates in humans compared to other staphylococcus strains (Price et al., 2012).

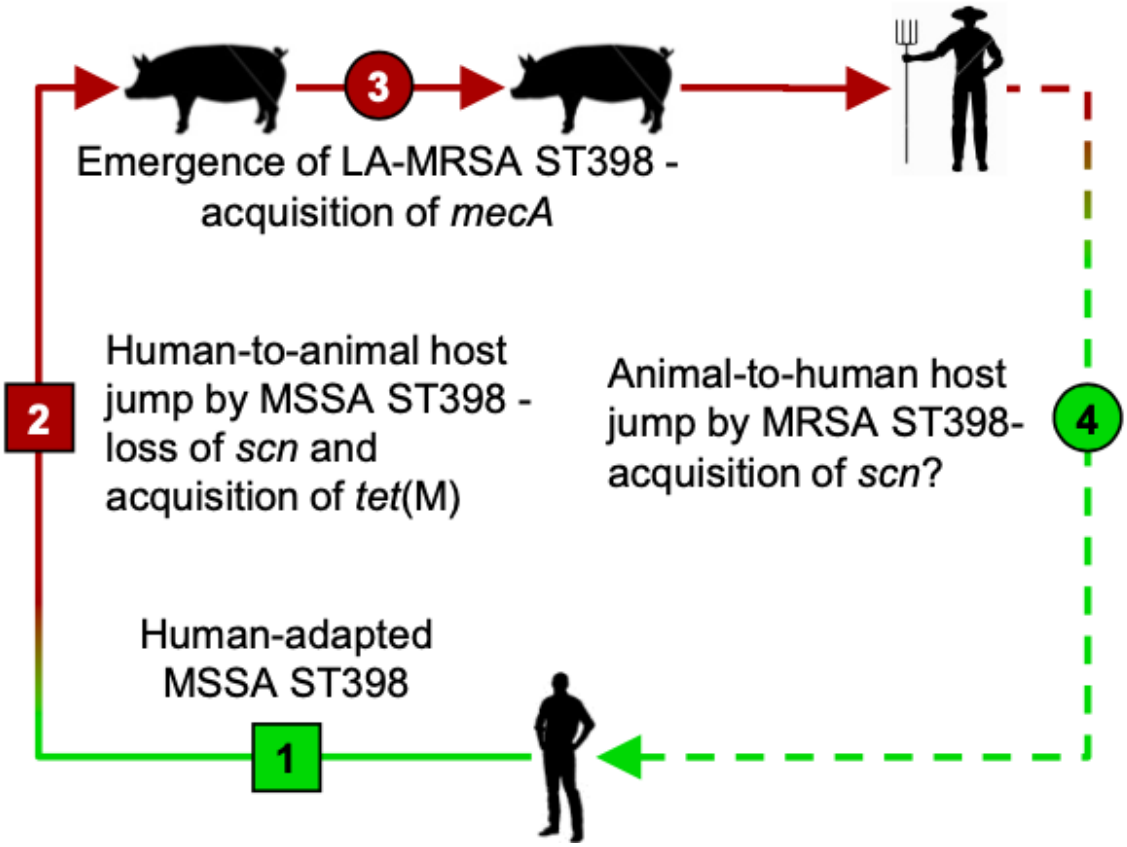


Figure 3. Showing the possible emergence of LA-MRSA CC398 (DANMAP, 2012)

## 4. Materials and methods

I obtained information and data for my thesis from various scientific published articles and journals. I used several search engines for collecting information, including “PubMed”, “Google Scholar”, “Scopus” and “Web of Science”.

Keywords used include “LA-MRSA”, “CC398”, “Methicillin-resistant *S. aureus*”, “Livestock”, “pig”, “swine”, “Denmark”, “prevalence”, “conventional”, “breeding”, “production”, “herd” among others, in a variety of combinations. I have included scientific papers from 1994 to 2021 in both English and Danish.

Additionally, I have collected data from Danish reports including DANMAP and Danish Veterinary and Food Administration.

# 5. Results and discussions

## 5.1 LA-MRSA in pigs in Denmark

### 5.1.1 Prevalence of LA-MRSA in pig herds

| Year | Number of breeding herds tested | % of breeding herds positive for MRSA | Number of production herds tested | % of production herds positive for MRSA |
|---|---|---|---|---|
| 2008 | 95 | 0% | 198 | 3,5% |
| 2010 | - | - | 99 | 16% |
| 2014 | 70 | 63% | 205 | 68% |
| 2016 | 6 | 100% | 221 | 88% |
| 2018 | 41 | 83% | 130 | 89% |
| 2019 | 73 | 95% | - | - |

Table 1. Overview of collected data on the estimated prevalence of LA-MRSA CC398 in breeding and production herds in Denmark from 2008-2019. (European Food Safety Authority, 2009; DANMAP, 2010; Danish Veterinary and Food Administration, 2014; Danish Veterinary and Food Administration, 2017b; DANMAP, 2020; Danish Veterinary and Food Administration, 2020; DANMAP, 2019).

### 5.1.2 Conventional pig herds

LA-MRSA was first identified in Danish pig farms in 2006. A survey done by European Food Safety Authority in 2008 showed that LA-MRSA was present at 0% of the tested breeding farms and 3,5% of the tested production farms in Denmark. In 2010, the percentage of positive production herds had increased to 16%. This indicates a significant rise in the prevalence of LA-MRSA, even though these two investigations varied in terms of the sampled populations (breeding herds versus production herds) and the collected samples (nasal versus dust samples) (Ciccolini et al., 2012).

Screening in 2014 showed that the occurrence of LA-MRSA positive herds increased significantly to more than 60% positive breeding and production herds, as seen on table 1 (Larsen et al., 2017). Danish Veterinary and Food Administration (DVFA) performed a screening in 2016 to investigate the prevalence of LA-MRSA. A total 221 different production herds and six breeding herds was included. The six breeding herds investigated were selected based on the results of the screening performed in 2014, where the result was that three of the selected herds tested positive and three tested negative. All six breeding herds tested positive in 2016, however, the number of investigated breeding herds in 2016 is too low to compare with the investigation done in the 70 breeding herds in 2014 (Danish Veterinary and Food Administration, 2017b).

Since the first screening of LA-MRSA was performed, the prevalence has increased to above 80% LA-MRSA positive herds in both the breeding and production sector. The increase of LA-MRSA among pig herds in Denmark is not desired since it poses a risk of spread to farmers, veterinarians, their close contacts and it increases the likelihood of further spread to the community (Sorensen et al., 2018).

Research has indicated that animal movement has a significant impact on the transmission and spread of LA-MRSA across pig herds, in addition to insufficient control measurements (Schulz et al., 2018). The structure of the pig production in Denmark is pyramidal, with the breeding herds including the nucleus and multiplier herd, at the top producing and selling sows to production farms at the base (Sieber et al., 2018). In respect of this, a possible explanation for the significant increase in positive pig herds in Denmark can be the spread

of LA-MRSA via the movement of pigs through the production pyramid. A study conducted by Van Duijkeren et al. in 2008 indicated that purchasing LA-MRSA positive pigs from another farm could result in colonization of the pigs (Van Duijkeren et al., 2008). As the spread of LA-MRSA is believed to occur via the pyramidal production chain primarily, herd size and trade connections could be substantial risk factors of the farm (Ciccolini et al., 2012).

There is a flow of pigs from a small number of breeding herds to a large number of production herds, and gilts are bought from other herds in most of the Danish integrated herds (Sorensen et al., 2017; Ciccolini et al., 2012). Positive breeding herds at the top provide a risk of transmitting LA-MRSA to the production herds at the base (Schulz et al., 2018). Furthermore, there is a possibility that LA-MRSA emerged in breeding farms between 2008-2014, and this has promoted its spread across the Danish swine production sector (Sieber et al., 2018).

LA-MRSA has been identified from the mucosa and skin of pigs, as well as from the surroundings, thus suggesting that transmission may occur directly or indirectly. Transmission between pigs in a herd is mostly via close contact. LA-MRSA can be present in dust and at environmental surfaces, contaminated fomites and means of transport. Hence this may act as a reservoir for LA-MRSA (Broens et al., 2012). Risk factors associated with LA-MRSA in pigs include the size of the herd, type of production and movement of pigs (Fromm et al., 2014). Schulz et al. 2018 aimed to investigate the fast expansion of LA-MRSA in pig herds from 2006 to 2015 in Denmark. Their Monte Carlo simulation model indicates that pig movement, indirect contact, and unexplained introduction of LA-MRSA in combination might account for the expansion of LA-MRSA seen in this period. However, it is purposed that neither of them could account for the expansion in Denmark solely on their own (Schulz et al., 2018).

The Danish breeding herds are closed herds with high infection control measures. The survey conducted in 2014 shows that 63% of the Danish breeding herds were positive for LA-MRSA, in comparison to 0% in 2008. This observation suggests that indirect routes of transmission other than pig trade affected the prevalence of LA-MRSA (Danish Veterinary and Food Administration, 2014).

### 5.1.3 Conventional versus alternative pig herds

In 2015, DVFA screened 64 organic pig herds out of a total of 95 organic pig herds in Denmark at that time. The 64 tested herds selected were those having more than 30 pigs. In total four out of the 64 herds tested positive, corresponding to an incidence of 6% (Danish Veterinary and Food Administration, 2016).

Later, in 2018, DVFA performed new LA-MRSA screening of alternative pig herds, (organic and free-range herds) and conventional pig herds (breeding and production herds). The result indicate that conventional pig herds had a higher prevalence of MRSA compared to organic and free-range pigs. They screened a total of 41 breeding herds and 130 production herds, where 83% of the breeding herds and 89% of the production herds tested positive. In comparison, the organic and free-range herds had considerably fewer positive cases, where the screening of 104 alternative pig herds revealed that only 20% tested positive. The majority of the isolates were spa types t034 and t011, where t034 was the most abundant type (DANMAP, 2019). Alternative pig herds may bring up to 20% of new breeding animals from conventional pig production per year, and this could be the gateway for LA-MRSA into these herds (Danish Veterinary and Food Administration, 2016).

Herds consisting of a greater number of pigs, such as conventional pig herds, may provide a larger risk of LA-MRSA contamination in contrast to small-sized herds, like alternative herds. Generally, the size of the herd is commonly identified as a risk factor related to infection and disease. The strong correlation between the size of the herd and risk of infection provides a greater chance of the introduction of infectious agents into the herd from the environment, which can be further transmitted and shed within the herd (European Food Safety Authority, 2010). It is believed that the reason behind fewer positive cases in organic and free-range pigs is the lower density of pigs within the herds, more space for each individual and less antimicrobial usage (DANMAP, 2019).

It is proposed that the prevalence of LA-MRSA is the greatest in growing pigs, and after the growth stage, the prevalence is reduced in negative correlation with age of fattening pigs (Fromm et al., 2014). In comparison to weaner-to-finish or grower-to-finish herds, farrow-

to-finish herds have a decreased probability of being LA-MRSA positive (Sorensen et al., 2018).

### 5.1.4 Norway versus Denmark

Denmark practices intense pig farming, composed of about 10.000 employees in the pig farms and approximately 30 million pigs produced yearly. Norway, on the other hand, has a smaller and not-so-intense pig production, bringing forth around 1.6 million slaughtered pigs yearly (Petersen et al., 2021).

In comparison to Denmark, multiple surveillance surveys performed in Norway showed that LA-MRSA was either absent or had a really limited prevalence among pig herds. During the European Food Safety Authority investigations in 2008, LA-MRSA was not detected in any of the investigated pig herds (Grontvedt et al., 2016). From 2013 to 2014, multiple different outbreaks of LA-MRSA took place in Norway. Following this, a stringent policy was implemented, which included the elimination of LA-MRSA by slaughtering pigs, annual surveillance programs, and decontamination of infected people. Up to this point, the policy has been effective, with no instances detected in the 872 tested pig herds in 2016 (Petersen et al., 2021). In light of this, stamping out served as a successful method to reduce the spread in this country (Schulz et al., 2019). Denmark, however, is in a situation now where the prevalence of LA-MRSA among the pig herds is much greater than it was in Norway at that time. Hence eradication in Denmark does not seem to be an option (Danish Veterinary and Food Administration, 2017a). Large-scale eradication and restocking of herds may raise ethical and economic concerns (Schulz et al., 2019).

There have been discussions about possible control measures for limiting LA-MRSA transmission among pig herds, including better hygienic conditions both during movement of pigs and within the pig herds and preventing pigs from LA-MRSA-positive farms from moving to LA-MRSA-negative herds (Schulz et al., 2019).

### 5.1.5 Action plan

In 2015 the Danish authorities published an action plan for the 4 upcoming years, with the primary aim to decrease the amount of both LA-MRSA positive pigs and herds, hence decreasing the risk of human colonization and infection. The action plan focused on 1) reduction of antibiotic usage, 2) hygiene measures with the aim of hindering the spread of LA-MRSA to the community by implementing measures including obligatory showering before entering and leaving the farm and hygiene course for employees handling pigs, 3) reduction of the spread of LA-MRSA within the herd by sectioning with effective hygiene measures and reduction of dust in the stable, 4) monitoring the evolution of LA-MRSA by screening herds every second year, with the purpose to evaluate the progress of LA-MRSA and the effect of the implemented measures, 5) further research about LA-MRSA (Schulz et al., 2019; Danish Veterinary and Food Administration, 2015).

## 5.2 LA-MRSA CC398 in Humans in Denmark

As of 2003, LA-MRSA has spread throughout the world. LA-MRSA is primarily associated with clonal complex 398 (CC398) and is particularly prevalent in pigs. MRSA CC398 has been detected in humans, most frequently in individuals who have had contact with pigs (DANMAP, 2014). The spread of LA-MRSA CC398 among pigs in Denmark has been a significant contributor to the rise in new human cases of MRSA (Petersen et al., 2021)

Occupational exposure poses a risk for human transmission, although there has been an increasing trend of human cases without livestock contact (Grontvedt et al., 2016).

### 5.2.1 Total LA-MRSA human cases

| Year | Number of CC398 cases | With livestock exposure | Without livestock exposure | Total MRSA cases |
|---|---|---|---|---|
| 2009 | 42 | - | - | 808 |
| 2010 | 111 | - | - | 1097 |
| 2011 | 164 | - | - | 1292 |
| 2012 | 232 | - | - | 1556 |
| 2013 | 643 | - | - | 2094 |
| 2014 | 1277 | - | - | 2965 |
| 2015 | 1122 | 982 | 140 | 2972 |
| 2016 | 1249 | 1040 | 209 | 3550 |
| 2017 | 1212 | 1019 | 193 | 3579 |
| 2018 | 1215 | 1057 | 158 | 3669 |
| 2019 | 1122 | 993 | 129 | 3657 |
| 2020 | 931 | 790 | 141 | 2883 |

Table 2. Overview of collected data on the estimated prevalence of positive MRSA CC398 cases in Humans from 2009-2020. (DANMAP, 2011; DANMAP, 2014; DANMAP, 2015; DANMAP, 2016; DANMAP, 2017; DANMAP, 2018; DANMAP, 2019; DANMAP, 2020; DANMAP, 2021)

From table 2, it is easy to see that the number of Danes who tested positive for LA-MRSA CC398 during the last decade has increased. The data collected is from Danish inhabitants who tested positive for LA-MRSA for the first time, either colonized or infected (DANMAP, 2018).

When a greater part of the population are carriers of LA-MRSA, the risk of elderly and immunocompromised persons being infected increases and may thus potentially cause fatal illness caused by LA-MRSA. Individuals testing positive for LA-MRSA have risen over the years, and it has been demonstrated that LA-MRSA can be transmitted from livestock to people, thus reducing the prevalence of LA-MRSA in pig herds may help to minimize the occurrence in humans (Schulz et al., 2019). It is therefore important to decrease the prevalence of LA-MRSA among the population, but measures must also take into consideration the fact that LA-MRSA seldom causes fatal illnesses in otherwise healthy individuals (Danish Veterinary and Food Administration, 2017a).

Denmark has witnessed a very rapid rise in positive MRSA cases from the beginning of 2012, and this is partly due to the emergence of LA-MRSA (Petersen et al., 2021). In 2013, the number of positive cases increased greatly, and this sudden rise of positive cases could be explained by the modified MRSA management recommendations released by the Danish Health and Medicines Authority in 2012, which recommended screening of MRSA for persons at risk of LA-MRSA CC398 carriage (primary or secondary livestock exposure) (Larsen et al., 2015). The modified recommendations aids to limit the spread of LA-MRSA into hospital and health care settings (Sieber et al., 2018).

The number of LA-MRSA CC398 positive cases continued to increase considerably to 1277 cases in 2014 and accounted for 43% of the total MRSA cases that year. The major part, comprising 89%, reported exposure to pigs (DANMAP, 2015). Individuals who work with or stay in close proximity to pigs would be at an elevated risk of colonization and infection with LA-MRSA (Ciccolini et al., 2012).

There was a decline in positive LA-MRSA CC398 cases, compared to the previous year, among Danes in 2015. This decline could be explained by the establishment of thorough screening of people having livestock contact, which may indicate a saturation point of this subpopulation, as a great part of the pig herds are positive for LA-MRSA, and the number

of employees in the pig industry is limited. The majority of positive LA-MRSA CC398 cases were from individuals who had been in contact with pigs (DANMAP, 2016).

Further, the COVID-19 restrictions led to a reduction in total MRSA positive cases which can possibly be a consequence of decreased international travel, social distancing, and decreased exposure to health care systems. From the positive cases reported, LA-MRSA CC398 accounted for 931 cases, which shows a reduction in LA-MRSA CC398 compared to the five previous years (DANMAP, 2021). The number of positive cases increased significantly until 2014 when the highest number was documented but has subsequently stabilized (Statens Serum Institut, 2020).

### 5.2.2 Infections

| Year | Number of infectious CC398 cases | With livestock exposure | Without livestock exposure |
|---|---|---|---|
| 2014 | 240 | | |
| 2015 | 208 | 131 | 77 |
| 2016 | 218 | 120 | 98 |
| 2017 | 272 | 174 | 98 |
| 2018 | 256 | 169 | 87 |
| 2019 | 253 | 167 | 86 |
| 2020 | 234 | 139 | 95 |

Table 3. Overview of collected data on the estimated prevalence of infectious LA-MRSA CC398 cases (DANMAP, 2015; DANMAP, 2016; DANMAP, 2017; DANMAP, 2018; DANMAP, 2019; DANMAP, 2020; DANMAP, 2021).

Skin and soft tissue infection are mostly related to LA-MRSA CC398 infections, and is mainly seen in young and healthy livestock employees, but it is also detected in the general population, including the elder and immunocompromised individuals being at a higher risk for developing severe illness (Larsen et al., 2017). LA-MRSA infections have resulted in invasive and life-threatening illnesses, for instance, bloodstream infections, and in some instances, death (Sieber et al., 2018).

Since 2014, the number of infections as a cause of LA-MRSA CC398 in Danish inhabitants with livestock exposure have maintained at a generally steady rate, with a range of 120 to 174 new positive cases yearly. Likewise, infections as a cause of LA-MRSA in Danish inhabitants without livestock exposure appears to have plateaued, composing 87 to 98 new positive cases reported between 2016 and 2018, as seen on table 3 above. However, these numbers exclude individuals who have tested positive for LA-MRSA CC398 within previous years (DANMAP, 2019).

The spreading of LA-MRSA CC398 throughout the production system of pigs in Denmark has been associated with an increased infection rate of Danes. In 2008, the first screening of LA-MRSA in pig herds in Denmark was performed. This showed a prevalence of 0% in the tested breeding herds and 3,5% in the tested production herds. Screening in 2014 showed that tested breeding and production herds had a prevalence of over 60% positive herds. In comparison to this, the number of infectious cases in humans gradually increased, reaching a high in 2014, as showed in figure 4 below (Sieber et al., 2018).

In 2014, LA-MRSA was responsible for 16% of bloodstream infections (BSI) and 21% of skin and soft tissue infections (SSTI) of the total MRSA BSI and SSTI cases that year (Larsen et al., 2017). The majority of Danes either colonized or infected are those who had livestock exposure. However, there is an increasing trend in infective cases without livestock exposure. If the incidence of LA-MRSA continues to expand into the unexposed community, this may lead to increased dissemination into hospitals (Larsen et al., 2015). Therefore, it is critical to maintain continual monitoring and efforts to avoid spreading of LA-MRSA from pigs to humans (DANMAP, 2019).

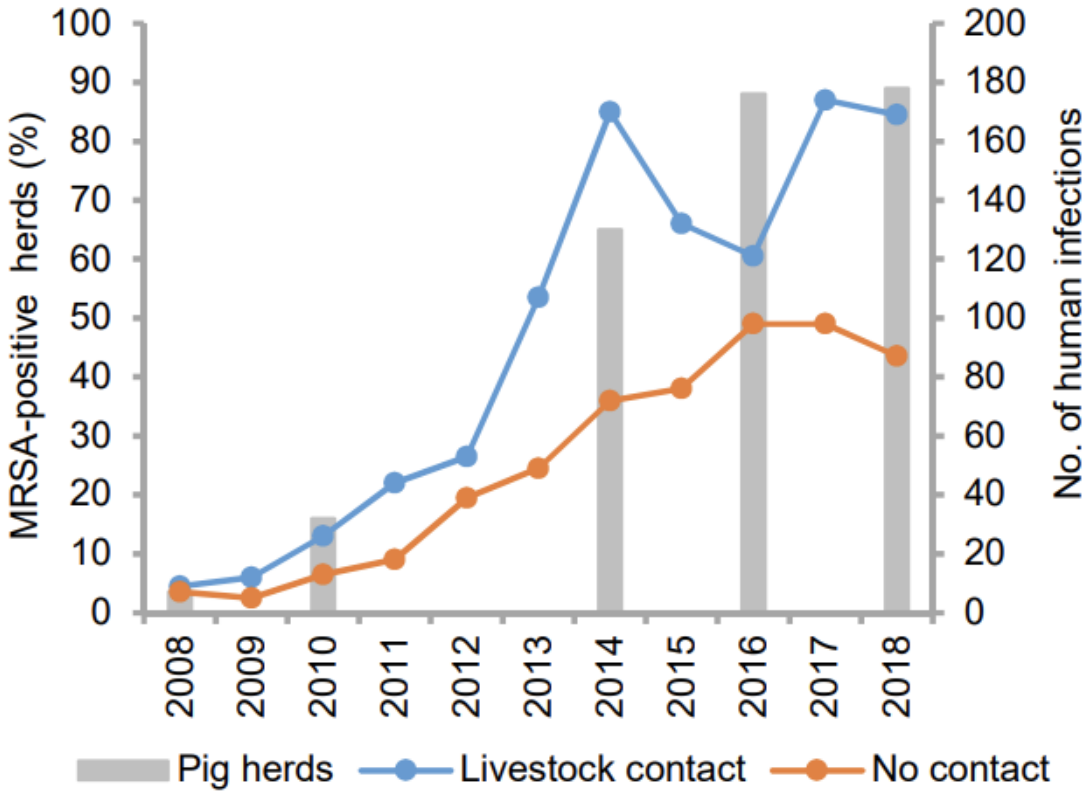


Figure 4. The relationship between the prevalence of LA-MRSA CC398 in pig herds and human infections with and without livestock exposure (DANMAP, 2019)

During the last years, infections in humans caused by LA-MRSA and LA-MRSA positive pig farms appears to have peaked, as seen on figure 4, this could imply that LA-MRSA colonization and infection in people might be a consequence of continual spread from pig farms (DANMAP, 2019).

## 6. Conclusion

The goal of my diploma work was to understand the importance of LA-MRSA in swine in Denmark. Over the last decade the unprecedented LA-spread across Danish pig herds has led to an increased risk of human colonization. Given the zoonotic properties of the LA-MRSA, the reservoir within the Danish pig population remains a threat to the public health system. Despite the fact that Denmark initiated a national action plan to decrease the amount of LA-MRSA in both pigs and herds, it has been a significant contributor to the rise of new human cases of MRSA.

It is indisputable that devoting proper preventive measures is high yield to combat the spread of LA-MRSA further. In spite of the already initiated action plan further control measures should be implemented with focus to reduce the prevalence of LA-MRSA within the Danish pig population, hence decreasing the risk of human colonization and infection.

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**Appendix 4.**

I hereby confirm that I am familiar with the content of the thesis entitled "Importance of Methicillin-Resistant *Staphylococcus aureus* in swine in Denmark" written by Emilie Aksdal Sorland which I deem suitable for submission and defence.

Date: Budapest, 16th November 2021

László Fodor
Department of Microbiology and Infectious Diseases

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