Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:60

Bovine Staphylococci and their Spectrum of Antimicrobial Resistance and Virulence Characteristics

Bovine stafylokokker og deres spekter av antimikrobiell resistens og virulensegenskaper

Marte Ragnhild Ekeland Fergestad

Bovine Staphylococci and their Spectrum of Antimicrobial Resistance and Virulence Characteristics

Bovine stafylokokker og deres spekter av antimikrobiell resistens og virulensegenskaper

Philosophiae Doctor (PhD) Thesis

Marte Ragnhild Ekeland Fergestad

Department of Paraclinical Sciences
Faculty of Veterinary Medicine
Norwegian University of Life Sciences

Oslo (2021)

Thesis number 2021:60
ISSN 1894-6402
ISBN 978-82-575-1832-5

Table of Contents

Acknowledgements

The work outlined in this thesis was carried out at the Department of Paraclinical sciences, Faculty of Veterinary Medicine at the Norwegian University of Life Sciences in the period 2016-2021. Six months were also spent at the research group for Molecular Microbiology (MolMik), Faculty of Chemistry, Biotechnology and Food Science.

I would like to express my gratitude to my supervisor team for invaluable guidance and support. I would especially like to thank my main supervisor Yngvild Wasteson for always believing in me, giving me freedom to find my own ways and answering all my questions, big and small. Your positivity, encouragement and support has meant a lot these past years. And a special thank you to my co-supervisor Morten Kjos for your patient introduction of a veterinarian into the world of molecular microbiology.

Many people have played a part in the creation of this thesis. Firstly, I would like to thank the team at MolMik for welcoming me into the lab like I had been there for years. You all made my stay very enjoyable and I learned a lot. And an extra thank you goes to Shian Zalehian for teaching and helping me in the lab. I would also like to thank my co-authors in the Anihwa group from Belgium and France for fruitful discussions and collaboration. I have truly appreciated our teamwork and annual meetings.

Special regards go to all my colleagues at Mattrygghet, with whom I have had the pleasure of working with for the last years. To my fellow phd-students, postdocs and research students, thank you for the laughter, chatter and long lunch talks. Thank you in particular to Nina Askim Vatne for all the laughter, shared troubles and discussions, whether it be what we actually should be discussing or revolving around dogs, clinics and other derailments. And a special thank you to Kristin

O’Sullivan for teaching me the ropes of the lab, your guidance and support have been very valuable.

It has been a long ride that would not have been the same without my family and friends. A big thank you to my family, whose constant encouragement, love and support has been with me every step of the way. To my friends, thank you for all the fun, laughter and cheering words. I know I have been even less social than usual lately, but I will try to up my game. A special thanks to my dear Håvard who has been my rock these last years. Thank you for patiently listening to all my thoughts and uncertainties. I could not have done this without you. And finally, I would like to thank my children, Sigrid and Eirik, who keeps me grounded and reminds me what is important (and who definitely delayed the completion of this thesis). I love you both very much.

Oslo, May 2021
Marte Ragnhild Ekeland Fergestad

Abbreviations

MRSA – Methicillin resistant *Staphylococcus aureus*

NAS – Non-*aureus* staphylococci

WGS – Whole genome sequencing

ECOFF – Epidemiological cut-off

EUCAST – European Committee on Antimicrobial Susceptibility Testing

HGT – Horizontal gene transfer

MGE – Mobile genetic element

CDC – Center for Disease Control and Prevention

WHO – World Health Organization

PCR – Polymerase chain reaction

PBP – Penicillin binding protein

List of papers

Paper 1

PBP2a provides variable levels of protection towards different β-lactams

M. E. Fergestad, G. A. Stamsås, D. M. Angeles, Zhian Salehian, Y. Wasteson, M. Kjos

MicrobiologyOpen. 2020 Aug;9(8):e1057

DOI: 10.1002/mbo3.1057

Paper 2

Antimicrobial resistance and virulence characteristics in three collections of staphylococci from bovine milk samples

M. E. Fergestad, A. De Visscher, T. L'Abee-Lund, C. Ngassam Tchamba, J. G. Mainil, D. Thiry, S. De Vliegher and Y. Wasteson

Journal of Dairy Science. 2021 Apr 29:S0022-0302(21)00568-3

DOI: 10.3168/jds.2020-19988

Paper 3

Whole genome sequencing of staphylococci isolated from bovine milk samples in Belgium and Norway

M. E. Fergestad, F. Touzain, S. De Vliegher, A. De Visscher, D. Thiry, C. Ngassam Tchamba, J.G. Mainil, T. L'Abee-Lund, Y. Wasteson and Y. Blanchard

Submitted to Frontiers in Microbiology

Summary

Staphylococci are a group of bacteria capable of colonizing and infecting a variety of host species and causing disease in a range of different tissues. *Staphylococcus aureus* is the most studied staphylococcal species and can cause both clinical and subclinical mastitis in bovines, as well as infections in a range of other species. This bacterium is associated with an array of virulence factors that contributes to its survival and spread. Non-*aureus* staphylococci (NAS) have, on the other hand, traditionally been regarded as a uniform group with an uncertain clinical importance, but in recent years they have emerged as the most frequently isolated bacterial group from bovine milk in many countries and they are increasingly associated with bovine udder infections. NAS are also regarded as a potential reservoir for antimicrobial resistance genes. Regarding the virulence of NAS, less is known about their content of virulence genes and their mechanisms for colonizing and infecting their hosts.

In addition to their large host and tissue range, staphylococci can acquire resistance to a variety of antimicrobial agents, of which methicillin resistant strains are of particular public health concern, especially methicillin resistant *Staphylococcus aureus* (MRSA). The term methicillin resistance incorporates resistance to almost all β-lactam antimicrobials, including penicillins and most cephalosporins. This resistance is mediated by the penicillin-binding protein PBP2a, encoded by *mec* genes, such as *mecA*.

To study the interaction between PBP2a and different β-lactam antimicrobials an IPTG-inducible *mecA* was introduced into a methicillin sensitive laboratory *S. aureus* strain. This confirmed that expression of PBP2a protects against β-lactam antimicrobials. By testing the resistance of the strain to a panel of nine different β-lactams, variations in the level of protection against different agents were shown. Microfluidics fluorescence time-lapse microscopy also demonstrated a considerable phenotypic variation between cells exposed to β-lactams. This also showed that *mecA*-expressing *S. aureus* can survive β-lactam concentrations considerably higher than the minimal inhibitory concentration.

As well as studying mechanisms related to the important methicillin resistance in staphylococci, the antimicrobial resistance and virulence characteristics in a collection of bovine staphylococci were examined, using disc diffusion, PCR and whole genome sequencing. The results showed that there was more antimicrobial resistance in NAS, compared to *S. aureus*, but the MRSA isolates stood out with a higher proportion of resistance characteristics compared to methicillin sensitive *S. aureus*. Regarding virulence, *S. aureus* contained a higher number of virulence genes compared to NAS. There were, however, also differences in virulence gene content between different NAS species, with species such as *Staphylococcus chromogenes* having a higher content of virulence genes compared to most other NAS species.

In summary, the findings in this thesis support the notion that there is great diversity within the genus *Staphylococcus*, not only between *S. aureus* and NAS, but also within the NAS and within a population of *mecA* expressing *S. aureus*. The results may have consequences for antimicrobial treatment of staphylococcal infections and show the need to tailor the choice of antimicrobial treatment regimen to the bacterial species in question and its antimicrobial resistance and virulence.

Sammendrag (Summary in Norwegian)

Stafylokokker er en gruppe bakterier som er i stand til å kolonisere og infisere både mennesker og mange forskjellige dyrearter, og bakteriene kan gi sykdom i flere ulike vev. *Staphylococcus aureus* er den mest studerte stafylokokkarten og kan forårsake både klinisk og subklinisk mastitt hos kyr. I tillegg kan *S. aureus* være årsak til ulike infeksjoner hos en rekke andre dyrearter. Dette skyldes ikke minst et stort utvalg av virulensfaktorer som bidrar til bakteriens overlevelse og spredning i verten. Non-*aureus* stafylokokker (NAS) har, i motsetning til *S. aureus*, tradisjonelt blitt ansett som en ensartet gruppe med usikker klinisk betydning. De senere år er imidlertid NAS rangert som de vanligste bakteriene isolert fra kumelk i flere land, og de blir stadig oftere satt i forbindelse med infeksjoner i juret hos melkekyr. NAS blir også ansett som et mulig reservoar for antimikrobielle resistensgener. Mindre er kjent om virulensen hos NAS, både når det gjelder innholdet av virulensgener og mekanismene for kolonisering og infeksjon av verten.

I tillegg til deres store verts- og vevsspekter kan stafylokokker tilegne seg resistens mot mange forskjellige antimikrobielle midler, hvor meticillinresistente stammer er spesielt utfordrende, særlig meticillinresistente *S. aureus* (MRSA). Begrepet meticillinresistens innbefatter resistens mot nesten alle β-laktamantibiotika, inkludert penicilliner og de fleste cefalosporiner. Resistensen skyldes det penicillin-bindende proteinet PBP2a, som er kodet av *mec* gener, blant annet *mecA*.

For å studere interaksjonen mellom PBP2a og forskjellige β-laktamantibiotika ble et IPTG indusertbart *mecA* gen satt inn i en meticillinsensitiv *S. aureus* laboratoriestamme. Dette bekreftet at ekspresjon av PBP2a beskytter mot β-laktamantibiotika. Ved å teste laboratoriestammens resistens mot ni forskjellige β-laktamer ble det konkludert med at graden av beskyttelse mot de forskjellige midlene varierte. «Microfluidics fluorescence time lapse»-mikroskopi viste en tydelig fenotypisk variasjon mellom celler som ble eksponert for β-laktamantibiotika. Dette viste også at *S. aureus* som uttrykker *mecA* kan

overleve β-laktam konsentrasjoner som er betydelig høyere enn minste inhibitoriske konsentrasjon.

I tillegg til å studere mekanismene knyttet til meticillinresistens i stafylokokker, ble antimikrobiell resistens og virulensegenskaper hos en samling bovine stafylokokker studert ved hjelp av disk diffusjon, PCR og helgenomsekvensering. Resultatene viste at NAS hadde en høyere andel antimikrobiell resistens sammenlignet med *S. aureus*, men MRSA isolatene skilte seg ut i *S. aureus* gruppen med en høyere andel resistens sammenlignet med meticillinsensitive *S. aureus*. Når det gjelder virulens hadde *S. aureus* en høyere andel virulensgener sammenlignet med NAS. Det var imidlertid også forskjeller i innholdet av virulensgener i forskjellige NAS arter. For eksempel hadde *Staphylococcus chromogenes* en høyere andel virulensgener sammenlignet med flere andre NAS arter.

Funnene i denne avhandlingen støtter antakelsen om at det et stort mangfold innen genuset *Staphylococcus*, ikke bare mellom *S. aureus* og NAS, men også innad i NAS eller i en populasjon av *S. aureus* som uttrykker *mecA*. Resultatene kan ha betydning for antimikrobiell behandling av stafylokokkinfeksjoner og viser behovet for skreddersydde antimikrobielle behandlingsregimer som tar hensyn til den aktuelle bakteriearten og dens resistens og virulens.

1. Introduction

1.1 One Health

With a growing human population and increasing globalization, humans and animals all over the world are connected directly and indirectly through food, water and soil. This close connection entails a great potential for exchange of microorganisms between species and between organisms and the environment.

Regarding health, this interconnection between human, animals and their environment is summed up in the term One Health. The World Health Organization (WHO) defines One Health as "*an approach to designing and implementing programmes, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes*" (WHO, 2017a), while the One Health Commission defines One Health as "*a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment*" (OHC, 2021).

Several microbial agents can infect both animals and humans and many are zoonotic and capable of transmitting between animals and humans (WHO, 2020b). In recent decades, there has been a rise in emerging infectious disease events with presumed animal origin, such as avian flu, SARS, AIDS and the very real example we have seen this last year of covid-19 (Taylor et al., 2001; WHO, 2020a). Changes in demography, economy and environment are considered to be among the major drivers of emerging infections (Jones et al., 2008). A growing human population causes people to live closer together and populate new areas, with effects such as deforestation and urban and agricultural expansion. This in turn leads to reduction and fragmentation of habitats for wild animals, creating new connections between wild animals and humans and domesticated animals and increasing the risk of animal to human transmission of infective agents (Patz et al., 2004; Weiss and McMichael, 2004). The population growth, increasing population density and

increasing globalization further provide a golden opportunity for onward spread of diseases (Weiss and McMichael, 2004).

The majority of the emerging or re-emerging diseases are bacterial or rickettsial, typically represented by antimicrobial resistant bacteria (Jones et al., 2008), such as methicillin resistant *S. aureus* (MRSA) and extended spectrum betalactamase (ESBL) producing *Enterobacteriaceae*. Often referred to as the silent pandemic, antimicrobial resistance is threatening both the human and animal health (Jasovský et al., 2016; Sharma et al., 2017). This pandemic is slow-moving and less visible, compared to e.g. covid-19 which suddenly caused a great impact on people's lives all over the world. Even so, an estimated 700 000 people die every year due to drug resistant microbes and it is assumed that with no actions taken the deaths attributed to antimicrobial resistance could rise to a staggering 10 million every year by the year 2050 (O'Neill, 2016).

The use of antimicrobial agents is considered the main driver for the development and dissemination of antimicrobial resistance (Tacconelli, 2009; Landers et al., 2012; Bell et al., 2014). Many of the antimicrobial agents used in veterinary medicine are also used in human medicine (ECDC et al., 2017) and several bacteria can cross species boundaries (Seguin et al., 1999; Lee, 2003; Hammerum and Heuer, 2009; Ferrari et al., 2019). There is evidence that bacteria transmitted from animal reservoirs can harbor antimicrobial resistance genes that confer resistance to important antimicrobial agents in human medicine (Wendlandt et al., 2015). There is also a skewed distribution of antimicrobials, where large amounts are used for humans and animals who do not need antimicrobial therapy, while others who need them do not have access (O'Neill, 2016). Given the close contact between animals and humans, the bacteria's ability to transmit between species and resistance genes to transfer between bacteria, antimicrobial resistance should be considered a shared problem that emphasizes the need for a One Health approach.

In the perspective of One Health, staphylococci are an important group of bacteria. They are found in numerous animal species and in humans, and studies have shown transmission of staphylococci from animals to humans and *vice versa*

(Seguin et al., 1999; Lee, 2013; Aires-de-Sousa, 2017). The most studied staphylococcal species is *Staphylococcus aureus*, a bacterium that can cause a wide range of diseases in both humans and animals, as well as living as a commensal on skin and mucus membranes. Staphylococci are also known to hold a variety of resistance determinants that in different combinations can give rise to multidrug resistant isolates (Kadlec et al., 2012; Wendlandt et al., 2015; Schoenfelder et al., 2017). Special attention is given to methicillin resistant *S. aureus* (MRSA) that are considered a high priority pathogen by the WHO (WHO, 2017b).

1.2. Staphylococci

Alexander Ogston first discovered staphylococcal bacteria, and in 1883 he named these cocci-forming organisms *Staphylococcus* (Ogston, 1882). The name originates from the Greek word Staphyle for "a bunch of grapes". The *Staphylococcus* genus belongs to the order *Bacillales* and the family *Staphylococcaceae*. Staphylococci are Gram-positive cocci, approximately 1 μm in diameter. The bacterial cells usually form grapelike, irregular clusters, hence the name. They appear as white to yellow, round, convex colonies on blood agar, varying from 0.5 to 4 mm in diameter, depending on the species, and are either beta-hemolytic or non-hemolytic.

1.2.1. Identification of staphylococci

Traditionally, methods using sugar fermentation and enzymes, such as catalase, have been used to distinguish staphylococci from other Gram-positive cocci. The standard coagulase test will determine if the bacteria are capable of coagulating plasma and can be used to separate the staphylococci into the coagulase positive or negative group (although there can be some variation in these results (Dos Santos et al., 2016)). Testing for production of the enzyme beta-galactosidase, the ONPG-test (o-nitro-phenyl-beta-galactopyranosid), and testing if the bacteria can cleave the carbohydrate mannitol, distinguish the two coagulase positive staphylococci *S. aureus* and *Staphylococcus pseudintermedius* (Vos et al., 2009).

Characterization of the genus *Staphylococcus* has revealed over 50 different species and subspecies (De Buck et al., 2021). They have historically been classified as coagulase positive or negative according to their ability to coagulate plasma *in vitro*, with *S. aureus* as the most common coagulase positive staphylococci. In recent years, the term non-*aureus* staphylococci (NAS) has emerged as an alternative expression for the coagulase negative staphylococci (CoNS), due to evidence that some species traditionally classified as CoNS are able to coagulate plasma (Dos Santos et al., 2016).

S. aureus is the most studied species of staphylococci, while NAS were previously considered to be minor pathogens and have traditionally been treated as a uniform group. One exception is *S. pseudintermedius*, who does not fit the general description of NAS, as this species is coagulase-positive and an important opportunistic pathogen in dogs (Bannoehr and Guardabassi, 2012), but will by definition be included into the NAS category. In the later years, NAS has emerged as a common cause of nosocomial infections in humans as well as subclinical mastitis in cattle (Pyörälä and Taponen, 2009; Becker et al., 2014b). The concept of NAS as a uniform group is becoming outdated and the notion that there are several differences between the NAS species is increasingly obvious (Supré et al., 2011; Vanderhaeghen et al., 2014; Condas et al., 2017b).

As the view on NAS is changing, several newer identification methods have been developed. Studies have shown that genotypic identification of NAS of bovine origin, such as sequencing of the *rpoB* gene (Supré et al., 2009), is overall a more accurate method compared to phenotypic methods. Commercial identification kits, such as API Staph ID 32 (API Test, bioMérieux, Lyon, France) that use miniaturized versions of different biochemical tests, are based on data from previously characterized human isolates and interpretation of results from both kits and conventional phenotypic tests is subjective and therefore challenging to compare between laboratories (Capurro et al., 2009; Sampimon et al., 2009; Zadoks and Watts, 2009). However, MALDI-TOF mass spectrometry has been shown to be a reliable tool for identification of NAS, but for bovine isolates an expanded

database is recommenced (Becker et al., 2014b; Cameron et al., 2017; Cameron et al., 2018).

1.2.2. Typing of staphylococci

There are several typing methods for determining strain affiliation for staphylococci. Most are developed for *S. aureus*, although there are schemes established for some of the more common NAS as well. Commonly used methods include pulsed-field gel electrophoresis (PFGE) and sequence-based typing such as staphylococcal protein A typing (*spa* typing) and multilocus sequence typing (MLST). The most common typing methods for staphylococci are presented in Table 1.

PFGE was previously the gold standard of bacterial typing. For staphylococci it relies on whole genome digestion with the restriction enzyme SmaI, followed by separation of the DNA segments on agarose gel. The method has a high discriminatory power and is suitable for identifying new strains new strains (Harmsen et al., 2003; Chambers and Deleo, 2009). However, the method is both time-consuming and laborious (Rodriguez et al., 2015) and results are sometimes difficult to compare across laboratories (Murchan et al., 2003).

The sequence-based methods are today more frequently used because they are easy to perform, data can easily be exchanged and results are reproducible between laboratories (Strommenger et al., 2008). MLST is based on single nucleotide variations within a selection of housekeeping genes, providing a sequence type (ST) (Chambers and Deleo, 2009) and is available for several NAS species as well as *S. aureus* (PubMLST, 2020). The method is highly reproducible, and, because it registers variation that accumulates slowly, it can be used to track evolution among lineages (Chambers and Deleo, 2009). *spa* typing is based on sequence analysis of variable number tandem repeats in the polymorphic X region of the protein A encoding gene, *spa*, and accounts for both point mutations in the region, as well as the number of repeat variations (Shopsin et al., 1999; Chambers and Deleo, 2009). The method combines many of the benefits of MLST, but is

faster, more practical and less costly in outbreak investigations because it only involves a single locus (Shopsin et al., 1999).

In addition to these methods, whole genome sequencing (WGS) is an increasingly used technique with a range of possibilities. WGS surpasses both PFGE, MLST and *spa* typing in terms of discriminatory power. This also means that WGS can divide previously identified clusters into sub-clusters, that may or may not be relevant (Rossen et al., 2018), depending on what resolution or level of detail that is appropriate for the investigation in question. With WGS it is possible to extract e.g. *spa*- and MLST-types *in silico*, and hence compare with results from these methods (Williamson et al., 2015; Rossen et al., 2018).

Table 1. Common methods for typing of staphylococci.

Method	Principle	Advantages	Disadvantages
Pulsed-field gel electrophoresis (PFGE)	Whole genome digestion with restriction enzyme SmaI followed by separation of DNA segments on agarose gel	High discriminatory power Identifying new strains Useful in outbreak investigations	Time-consuming Laborious Results difficult to compare between laboratories
Multilocus sequence typing (MLST)	Single nucleotide variation within a selection of housekeeping genes, providing a sequence type (ST)	Highly reproducible between laboratories Useful for tracking evolution among lineages	Not as useful for outbreak investigation Costly (but becoming less expensive)
***spa*-typing**	Sequence analysis of tandem repeats in X-region of the *spa* gene. Providing a *spa*-type	Fast and practical Less costly than MLST Useful in outbreak investigations	Not as useful for tracking evolution over a longer time period
Whole genome sequencing (WGS)	DNA sequencing of the complete genome of a species	Wide range of possibilities Very high discriminatory power Possible to infer *spa*- and MLST-types *in silico* Useful in outbreak investigations	Costly (but becoming less expensive) High resolution can divide clusters into irrelevant subclusters

1.2.3. Habitat, host specificity and clinical significance

Staphylococci occur worldwide as commensals on the skin and mucus membranes of humans and animals, and they cause a broad range of infections. Different animal species are associated with different staphylococcal species, but several staphylococci can colonize and infect both humans and animals (Lowy, 1998; Trostle et al., 2001; Otto, 2009; Becker et al., 2014b; Artursson et al., 2016). Figure 1 shows different staphylococcal species associated with humans, animals or both.

S. aureus is considered a multi-host "generalist" capable of causing an array of different diseases in a range of different host species (Sheppard et al., 2018). However, some individual lineages of *S. aureus* have also developed host-specialization (Lowder et al., 2009; Fitzgerald, 2012). Cell wall-associated proteins are thought to be important for host specificity (Pickering and Fitzgerald, 2020).

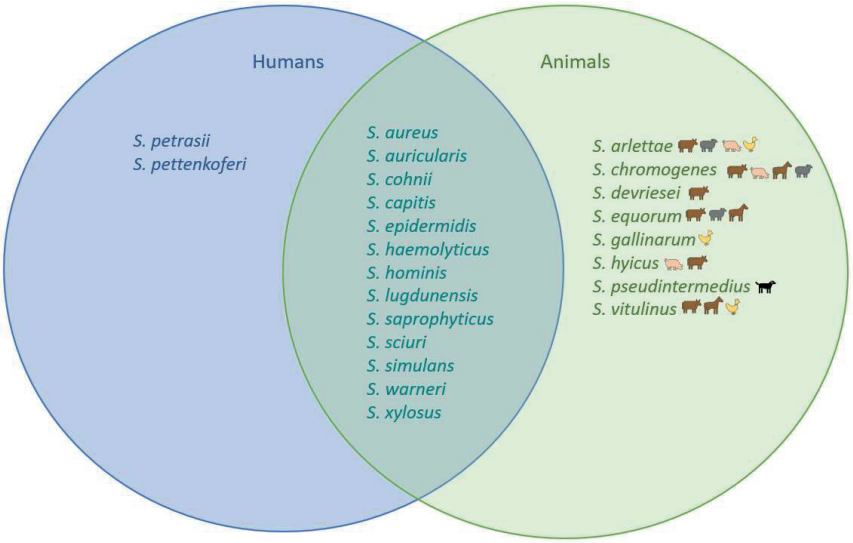


Figure 1. Common staphylococcal species isolated from humans, animals, and both. The animal species typically associated with staphylococcal species isolated from animals are indicated with different icons.

Staphylococci in humans

Approximately 30 % of the human population are asymptomatic carriers of *S. aureus*, commonly found at sites such as the nares, throat and perineum (Sollid et al., 2014). Besides being a commensal *S. aureus* is also a major human pathogen and an important cause of skin and soft tissue infections, bacteremia and infective endocarditis, and osteoarticular and device related infections (Josse et al., 2015; Thammavongsa et al., 2015; Tong et al., 2015; Walker et al., 2017). In relation to this, MRSA is of special concern and it is common to separate MRSA infections in humans into healthcare associated MRSA (HA-MRSA) and community associated MRSA (CA-MRSA), often represented by different strains (Mediavilla et al., 2012). HA-MRSA is usually associated with severe invasive disease in hospitalized patients, while CA-MRSA occurs in the absence of healthcare exposure, but the distinction between HA- and CA-MRSA is becoming gradually more blurred (Mediavilla et al., 2012; Choo, 2017).

In an analysis of the microbiome of the human nose several NAS species were identified, with *S. epidermidis* colonizing virtually all patients (97 %) (Kaspar et al., 2016). As for infections with NAS, in an immunocompetent human, urinary tract infection with *Staphylococcus saprophyticus* and NAS endocarditis are most common. However, far more common are infections in immunocompromised patients and these are often device related (Piette and Verschraegen, 2009; Soumya et al., 2017). NAS accounts for up to 30 % of all clinically relevant nosocomial bloodstream infections in humans, the majority being catheter-related sepsis (Piette and Verschraegen, 2009). Overall *S. epidermidis* is the most common NAS species causing infections in humans, followed by *Staphylococcus hominis*, *S. haemolyticus* and *Staphylococcus capitis* (Becker et al., 2014b).

Staphylococci in animals

Many staphylococcal species are found as commensals and opportunistic pathogens in animals. *S. aureus* is a well-known udder pathogen in dairy cows causing both clinical and subclinical mastitis (Osterås et al., 2006; Olde Riekerink et al., 2008),

while NAS are the most common cause of bovine subclinical mastitis, with *Staphylococcus chromogenes* as the most prevalent species (De Visscher et al., 2016; Condas et al., 2017a). As *S. chromogenes* is an important cause of subclinical mastitis and rarely isolated from the environment in the barn, it is assumed that the species has a host adapted ecology (Piessens et al., 2011; De Visscher et al., 2016). Other bovine associated NAS species, such as *Staphylococcus cohnii, Staphylococcus equorum* and *Staphylococcus sciuri*, are more frequently isolated from the environment, pointing to an environmental nature (Piessens et al., 2011; De Visscher et al., 2016), although these species have also been isolated from the bovine udder (Condas et al., 2017b).

S. aureus is also often found on pig farms (Mroczkowska et al., 2017; Feld et al., 2018). During the last decade, livestock-associated MRSA (LA-MRSA) has become an increasing concern. LA-MRSA are associated with animals and most notably pig holdings, and the strains often belong to one lineage (clonal complex 398), although other lineages have been reported (Elstrøm et al., 2019). Pigs are considered an important reservoir and LA-MRSA is found on pig farms in several countries (Sieber et al., 2018; Sørensen et al., 2018; Elstrøm et al., 2019; Merialdi et al., 2019), where it has received considerable attention because of its significance for public health. The pigs are usually asymptomatic carriers. LA-MRSA has also been reported from dairy farms, although it is speculated that this might be spillover from pig production (Hansen et al., 2019). LA-MRSA have colonized and caused disease in humans, both people working with livestock (Bosch et al., 2015), but also in people with no livestock exposure (Bosch et al., 2016) and LA-MRSA is now an important cause of human infection in countries such as Denmark and the Netherlands (Sieber et al., 2018).

Both *S. aureus* and NAS have been found in sports- and companion animals, such as dogs, cats and horses (Couto et al., 2016). In dogs, *S. pseudintermedius* is an important commensal and opportunistic pathogen (Bannoehr and Guardabassi, 2012) and there are also examples of this species, especially methicillin resistant strains (MRSP), causing disease in humans (Stegmann et al., 2010; Starlander et al., 2014).

1.2.4. Pathogenesis and virulence

Pathogenesis and virulence in S. aureus

S. aureus is a well-adapted pathogen capable of inhabiting and causing disease in a variety of body sites in both humans and animals, largely due to a broad range of virulence factors that manipulate the host's immune responses (Tam and Torres, 2019). The bacteria can colonize and infect many different tissues from skin to deeper structures, for example the heart and bones and cause serious conditions as bacteremia and sepsis (Jenkins et al., 2015; Balasubramanian et al., 2017).

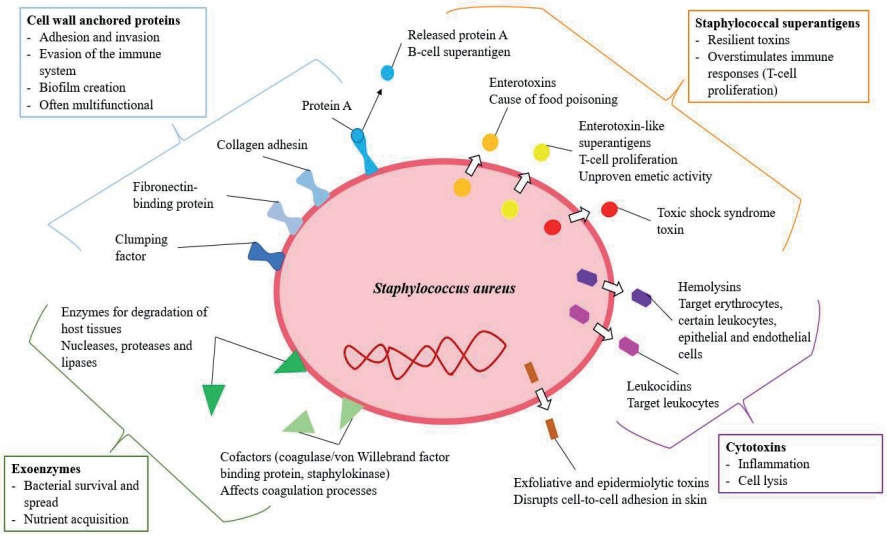


Figure 2. Overview of the main groups of virulence factors in *S. aureus*.

Transitioning from a commensal to an invasive pathogen requires a great deal of adaption, where the bacteria must adjust virulence in response to the host environment. In the early stages of infection *S. aureus* produces a range of cell wall associated factors enabling adhesion and evasion of host immune responses, allowing for growth and biofilm formation (Kong et al., 2006; Schilcher and Horswill, 2020; Kranjec et al., 2021). Depending on the cell density and

environmental conditions, cells may detach from the biofilm, potentially leading to dispersal of the infection. At the same time, the cells may also express an array of exotoxins, leading to dissolution of biofilm and amplification of spread (Dinges et al., 2000). The regulation of *S. aureus* virulence is based on a complex network of regulatory mechanisms, recognizing environmental signals and activating master regulators acting to modify gene expression (Balasubramanian et al., 2017). The transition between the adhesion phase and the invasion phase largely depends on the intercellular communication process of quorum sensing, where a constitutively produced molecule, a so-called autoinducer, which act as an indicator of population density (Wang and Muir, 2016). Quorum sensing enables the bacteria to monitor the environment for other bacteria and modify responses at population level based on changes in cell densities in the surroundings (Waters and Bassler, 2005). The chromosomal locus *agr* is responsible for both producing and detecting the autoinducer and for transmitting the signal intracellularly to a transcriptional regulator that eventually controls the virulence gene expression (Waters and Bassler, 2005; Wang and Muir, 2016).

The broad spectrum of virulence factors produced by *S. aureus* includes both cell wall anchored proteins, toxins, and extracellular enzymes (Figure 2). The cell wall anchored proteins have several roles, such as adhesion to and invasion of host cells and tissue, evasion of the immune system and creating biofilm (Foster et al., 2014; Geoghegan and Foster, 2017). The exact composition of these surface proteins differs between strains (McCarthy and Lindsay, 2010). The predominant group of cell wall anchored proteins is the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family. In *S. aureus* this group includes among others, clumping factor A (ClfA) and ClfB binding fibrinogen, fibronectin binding protein A (FnBPA) and FnBPB, binding fibronectin and collagen adhesin (Can) binding collagen. Protein A, the coding region for which is used in *spa* typing, is also part of this group (Foster et al., 2014). In addition to the fibronectin binding function, FnBPs are also involved in adhesion to and internalization into mammalian cells (Dziewanowska et al., 1999; Sinha et al., 1999). Internalization and survival in neutrophils can promote spreading of infection and uptake into non-

phagocytic cells can damage host cells, as well as protecting the bacteria from both the host immune system and antimicrobial therapy (Sendi and Proctor, 2009; Thwaites and Gant, 2011). Several of the cell wall anchored proteins inhibit immune responses by altering antigens on the bacterial surface, hindering complement activation and promoting degradation of complement factors (Hair et al., 2010; Kang et al., 2013; Foster et al., 2014). This means that these cell wall anchored proteins have a variety of functions that are crucial for colonizing and invading the host and are essential for bacterial survival both in the commensal state and during infection (Foster et al., 2014). In the bovine udder, FnBPs play an important role in adhesion and invasion (Lammers et al., 1999), along with ClfA and ClfB, which are also though to contribute to the initial adherence to the teat canal (da Costa et al., 2014).

S. aureus produces a broad range of secreted toxins and exoenzymes. Toxins, such as cytotoxins and superantigens, promote inflammation and leukocyte cell death, while exoenzymes cleave and disable several immune molecules (Tam and Torres, 2019). Cytotoxins consist largely of pore-forming toxins. The archetypal pore-forming toxin α-toxin or α-hemolysin (hla), encoded by the *hla* gene, causes lysis of numerous cell types, including erythrocytes, endothelial cells, epithelial cells and certain leukocytes (Grimminger et al., 1997; Inoshima et al., 2011; Nygaard et al., 2012). The bicomponent pore-forming toxins share a structural homology and formation mechanism with α-toxin, but primarily target leukocytes, hence they are also called leukocidins (Luk) (Tam and Torres, 2019). Some of the leukocidins, such as LukMF, have a limited host species range, but are important in ruminants (Abril et al., 2020).

Staphylococcal superantigens (SAgs), the largest group of exotoxins produced by *S. aureus*, are resilient toxins, resistant to heat, proteolysis and desiccation (Spaulding et al., 2013; Tam and Torres, 2019). SAgs overstimulate many immune responses and can induce large T cell proliferation followed by a lack of T cell immune response where activated T cells fail to multiply and might undergo apoptosis (Spaulding et al., 2013; Tam and Torres, 2019). Because of their severe toxicity and resilient nature some superantigens are classified as potential

agents for bioterrorism (Spaulding et al., 2013). These toxins comprise several groups; staphylococcal enterotoxins (SEs), SEA to SEE and SEG, capable of causing emesis and diarrhea, staphylococcal enterotoxin-like superantigens (SE-ls), SE-lH to SE-lX that induce T cell proliferation, but have unproven emetic activity and toxic shock syndrome toxin-1 (TSST-1) responsible for the severe, acute, systemic disease known as toxic shock syndrome. B cell superantigen, for which staphylococcal protein A is the only known representative, that includes release of protein A from the cell wall followed by binding to immunoglobulins to prevent opsonization (Tam and Torres, 2019) is also included in these toxins. The role of superantigens in the pathogenesis of bovine mastitis is not fully known. One study have indicated that these proteins contribute in staphylococcal udder infection by promoting tissue damage and inflammation, as well as immunosuppression and immune evasion (Wilson et al., 2018). Cytotoxic enzymes, such as β-toxin or β-hemolysin (hlb) cause species-dependent hemolytic activity. In bovine *S. aureus* isolates the *hlb* gene appears more common than *hla* (Aarestrup et al., 1999). While in human isolates studies have shown *hla* to be more common (Aarestrup et al., 1999; Booth et al., 2001). The hlb, encoded by a lysogenic bacteriophage, can damage keratinocytes, aiding in bacterial colonization of mammalian skin (Abril et al., 2020). Exfoliative toxins or epidermolytic toxins disrupt the cell-to-cell adhesion in the top layer of the skin, resulting in blistering and desquamation (Tam and Torres, 2019).

Exoenzymes are another group of secreted virulence factors that contribute to bacterial survival and spread, as well as nutrient acquisition. This group can be divided into cofactors that activate host zymogens and enzymes for degradation of host tissue (Tam and Torres, 2019). Cofactors, such as coagulase, von Willebrand factor binding protein and staphylokinase, work by hijacking different processes in the host coagulation system, affecting immune responses and stimulating bacterial survival and dissemination (Bjerketorp et al., 2004; McAdow et al., 2012; Nguyen and Vogel, 2016). Finally, *S. aureus* produces a wide range of nucleases, proteases and lipases that degrade host tissue components for gaining nutrients and evade host immune responses (Tam and Torres, 2019).

Pathogenesis and virulence in NAS

While *S. aureus* is associated with a range of virulence factors, less is known about the pathogenesis and virulence factors in NAS (Becker et al., 2014b; Naushad et al., 2019). NAS have previously been considered only as commensals in both human and veterinary medicine. The emergence of nosocomial NAS infections in humans and the fact that NAS are the most frequently isolated bacteria in bovine subclinical mastitis in many countries (Becker et al., 2014b; De Visscher et al., 2017) have led to a change in the view of these species as simple commensals. The importance of NAS as udder pathogens in bovines is debated. They are linked to increased somatic cell count (De Vliegher et al., 2012; De Visscher et al., 2016; Valckenier et al., 2019), though there are diverging reports on the effect on milk production (Heikkilä et al., 2018; Valckenier et al., 2019). Most NAS species have similar regulatory mechanisms as *S. aureus*, such as the *agr* system (Becker et al., 2014b). Although many virulence genes have been identified in NAS there is not always a simple link between the number of virulence genes and severity of disease (Naushad et al., 2019). While *S. aureus* can produce as many as 24 different cell wall anchored protein, NAS such as *S. epidermidis* and *Staphylococcus lugdunensis* express far less (Bowden et al., 2005; Heilbronner et al., 2011). Breaching of the skin barrier is a crucial step in transitioning NAS species from commensals to pathogens, where many of the factors involved in the commensal lifestyle on the skin are turned into virulence factors (Argemi et al., 2019). In humans, NAS are capable of adhering to host cells or foreign body surfaces, such as indwelling medical devices, and creating biofilm by producing various adhesins, which are considered crucial features for NAS device associated infections. Different extracellular enzymes can then degrade the biofilm, causing dissemination of bacteria with the bloodstream. Like *S. aureus,* NAS may also be phagocytized by non-phagocytic cells where they can evade the immune system and antimicrobial therapy (Becker et al., 2014b). Bovine NAS has also been shown to carry a large variety of virulence genes, and exoenzymes have been detected in several bovine NAS species, as well as cytotoxin genes such as *hlb*, while very few leukocidins have been detected (Åvall-Jääskeläinen et al., 2018;

Naushad et al., 2019). Superantigen genes, such as genes encoding staphylococcal enterotoxins, have also been identified, while very few isolates harbor the toxic shock syndrome toxin *tsst-1* (Mahato et al., 2017; Naushad et al., 2019).

1.3. Antimicrobial resistance

1.3.1. Emergence and drivers of antimicrobial resistance

Antimicrobial resistance is a global challenge and threatens both animal and public health. The successful use of any antimicrobial agent, whether working against bacteria, fungi, parasites or viruses, is constantly threatened by the development of resistance (Davies and Davies, 2010).

Microorganisms have, through evolution, developed many antimicrobial substances to kill or inhibit neighboring cells in environments with a diversity of microbial species. With a few exceptions of fully synthetic drugs, most antimicrobial drugs used today are agents naturally produced by microorganisms or synthetic derivates of these. To defend themselves from these toxic substances microorganisms have also naturally evolved a range of protective mechanisms (Holmes et al., 2016). Environmental bacteria thus have a wide range of antimicrobial resistance genes (Linares et al., 2006; Aminov, 2009). Still, even though many environmental microorganisms naturally produce antimicrobials, there is little evidence that this significantly contributes to the selection of antimicrobial resistant microorganisms (Martinez, 2009). Instead the use of antimicrobials for humans, animals and agriculture is a major factor in driving antimicrobial resistance (Landers et al., 2012; Bell et al., 2014). For example, there is evidence that enzymes inactivating β-lactam antimicrobials, the β-lactamases, have existed for millions of years (Aminov, 2009). Thus, while penicillinase, the enzyme inactivating penicillin, was discovered soon after the discovery of penicillin in 1928, resistant strains were not common before the drug was in commercial use (Abraham and Chain, 1988; Davies and Davies, 2010). Compared to the pre-antibiotic era, the use of

antimicrobials in medicine and agriculture has increased the selection pressure dramatically, and this has accelerated the development and spread of antimicrobial resistant bacteria.

A large quantum of the antimicrobials used worldwide are prescribed for animals (WHO, 2012). In the US over 70 % (by weight) of medically important antibiotics for humans are sold for use in animals (O'Neill, 2016). Some are necessary for treating sick animals and securing animal welfare and food security, however, a substantial proportion is not used for these intentions, but rather used preventively or for growth promotion (Landers et al., 2012; O'Neill, 2016). This misuse of antimicrobials in animals is most likely playing a major role in the global emergence of antimicrobial resistance (WHO, 2012).

The link between the use of an antimicrobial agent and subsequent development and spread of resistance is not always straightforward. There are many factors that can affect this, such as interactions between the pathogen and the drug in question, interactions between pathogen and host, mutation rates of the pathogen, horizontal gene transfer, spread of pathogens among humans, animals and the environment, cross-resistance and co-selection of isolates resistant to other drug classes (Holmes et al., 2016). In fact, studies on *E. coli* in animals have revealed that treatment with penicillin can lead to increased phenotypic resistance to several unrelated antimicrobials (Grønvold et al., 2010; Grønvold et al., 2011). Besides, factors such as vaccination, migration and tourism, hygienic conditions and population densities also impact resistance prevalence (Holmes et al., 2016; Hendriksen et al., 2019).

1.3.2. Genetic basis of antimicrobial resistance

The antimicrobial resistance of bacteria can be divided into intrinsic and acquired resistance. Many bacteria are inherently resistant to different antimicrobials. These intrinsic mechanisms can be due to lack of target molecule or can be encoded within the genome of a bacterial species independent of any selective pressure or horizontal gene transfer (Cox and Wright, 2013). In addition to the intrinsic

mechanisms, bacteria can develop resistance. This can happen either through mutations in genes associated with the functionality of an antimicrobial substance or by acquisition of new genes through horizontal gene transfer (HGT), where the bacteria acquire foreign DNA material coding for resistance determinants (Munita and Arias, 2016). Resistance may thus occur in a bacterial cell in a susceptible cell population, creating a resistant sub-population. Antibiotic treatment will lead to eradication of the susceptible population and perseverance of the resistant bacteria (Munita and Arias, 2016).

HGT is one of the most important genetic drivers of dissemination of antimicrobial resistance, giving the bacteria a large adaptive capacity in their defense against antimicrobials. There are three main mechanisms of HGT, by which bacteria acquire foreign genetic material, namely conjugation, natural transformation, and transduction. In conjugation there is physical contact between the donor and the recipient cell and genetic material is transferred. In transformation, extracellular DNA is acquired from the surroundings, while transduction relies on transfer of genetic material via bacteriophages, due to integration of genetic material from the bacterial host into the phage genome (Soucy et al., 2015).

The basis for this horizontal gene transfer is mobile genetic elements (MGEs), which are DNA fragments that can move intercellularly and intracellularly, and thus can contribute to the accumulation and dissemination of resistance and virulence genes. MGEs consists of different elements with different sizes and properties, such as plasmids, integrative conjugative elements (ICE), bacteriophages, transposons and insertion sequences. An overview of different MGEs and their general properties are presented in Table 2.

Plasmids are transferred via conjugation and are important in horizontal gene transfer, carrying both other MGEs and antimicrobial resistance genes. Some plasmids can carry several resistance determinants, so-called multi-resistance plasmids (Malachowa and DeLeo, 2010; Schwarz et al., 2014; Partridge et al., 2018). They can replicate separately from the chromosome using replication genes, are usually distributed to daughter cells through cell division and some can be

transferred to other bacteria via horizontal gene transfer (Malachowa and DeLeo, 2010; Schwarz et al., 2014). Like plasmids, ICE can be transferred via conjugation, however they integrate into and are generally replicated as part of the host chromosome. ICE are a diverse group and can carry a variety of antimicrobial resistance genes (Partridge et al., 2018).

Insertion sequences (IS) and transposons (Tn) can move themselves and associated resistance genes around in the same or different DNA molecule within the bacterial cell. This includes moving between plasmids and between a plasmid and the genome. One IS elements can only move itself, but if two similar IS elements flank a resistance gene, they are able to move it together. This is called a composite transposon. The non-composite transposons are larger and more complex and can contain resistance genes. These transposons also have specific genes that enable them to move (Partridge et al., 2018). These elements move between cells via other MGEs, such as plasmids, ICE and phages (Frost et al., 2005). The distinction between IS and transposons could be difficult due to overlapping properties (Siguier et al., 2009).

Phages are mobile genetic elements responsible for transduction, but compared to other MGEs less is known about their contribution to the dissemination of antimicrobial resistance (Colomer-Lluch et al., 2011). However, it is been suggested that bacteriophages can contribute to horizontal transfer of antimicrobial resistance genes because phages containing antimicrobial resistance genes have been found in several environments, such as seawater (Blanco-Picazo et al., 2020), soil (Larrañaga et al., 2018) and the human intestine (Brown-Jaque et al., 2018). These bacteriophages may have the potential to mobilize these genes and contribute to transmission between bacterial communities.

In methicillin resistant staphylococci, the resistance island staphylococcal cassette chromosome *mec* (SCC*mec*), carrying the genes responsible for methicillin resistance, is also considered a mobile genetic element. Although the exact transfer mechanism of this element is still unclear, the element is considered a mobile genetic element because it contains recombinase genes, is genetically diverse. The occurrence of different SCC*mec* types in bacterial isolates belonging to the same

clonal lineage and in different staphylococcal species is taken as evidence that the element can be transferred between staphylococcal strains and between staphylococcal species (Smyth et al., 2011; Kriegeskorte and Peters, 2012). In addition, SCC*mec* carry replication genes, meaning they could potentially self-replicate, which would make horizontal transfer more efficient by providing multiple copies of the element before transfer (Mir-Sanchis et al., 2016), although transfer of the element is believed to occur relatively infrequently.

Table 2. Overview of common mobile genetic elements in staphylococci.

Mobile genetic element	Typical size	Description	Mechanism of intracellular DNA transfer	Content
Plasmids	1-1000 kb Average size in staphylococci 5-40 kb	Circular, self-replicating extra-chromosomal DNA molecules. Self-transmissible	Conjugation	Resistance determinants. Can encode toxin genes
Integrative conjugative elements (ICE)	13-500 kb	Integrates into and replicates as part of host chromosome. Self-transmissible	Conjugation	Resistance determinants
Bacteriophages (phages)	16-140 kb	Self-transmissible. Virus infecting bacteria. Use bacterial cellular machinery to reproduce. May transfer and incorporate bacterial host DNA	Transduction	Virulence and resistance determinants
Staphylococcal cassette chromosome *mec* (SCC*mec*)	21-67 kb	Resistance island carrying *mec* genes Presumed transmissible	Unknown	*mec* genes May contain other MGEs and other resistance determinants
Transposons	2-21 kb	Able to move between DNA molecules intracellularly	Via plasmids, phages or ICEs	Resistance determinants
Insertion sequences (IS)	1-2 kb	Able to move between DNA molecules intracellularly as part of composite transposon	As for transposons	Resistance determinants

1.3.3. Mechanisms of resistance

Bacteria can exhibit resistance to antimicrobials through different mechanisms, presented in Figure 3. These include mechanisms to prevent the antimicrobial from reaching their target by changing the permeability of the cell envelope (Fig a) or via efflux pumps (Fig b), modification of the antimicrobial target (Fig c) or enzymatic inactivation of the antimicrobial by addition of chemical groups (such as acetylation, Fig. d) or degradation of the molecule (Fig. e) (VKM et al., 2020). Some of these mechanisms will be further elaborated on in following paragraphs.

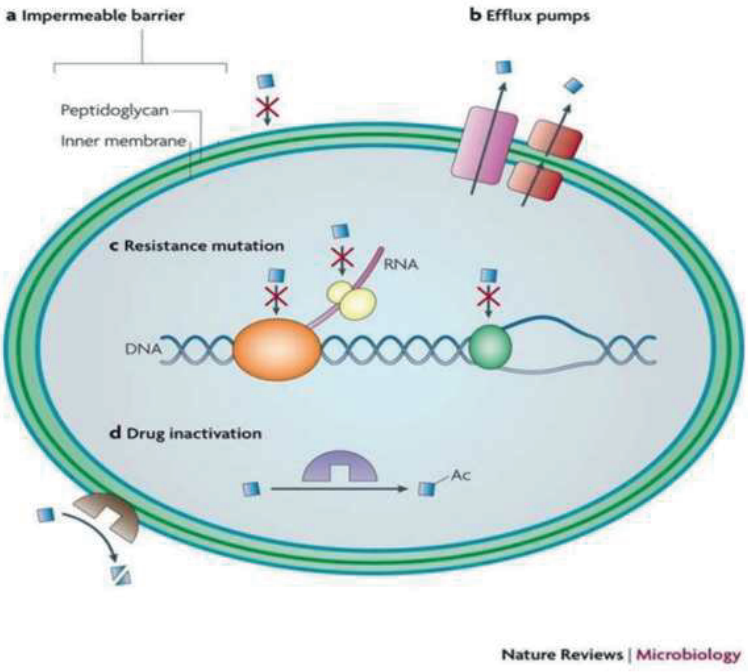


Figure 3. Overview of main antimicrobial resistance mechanisms. (Adapted with permission from Allen et al. 2010).

1.4. Antimicrobial resistance in staphylococci

Staphylococci are naturally susceptible to a broad range of antimicrobials, but they can acquire and carry genes conferring resistance to a wide variety of

antimicrobials. Figure 4 shows an overview of common antimicrobial agents, their modes of action and whether they are used in animals and/or humans. Table 3 shows an overview of common antimicrobial resistance genes in staphylococci.

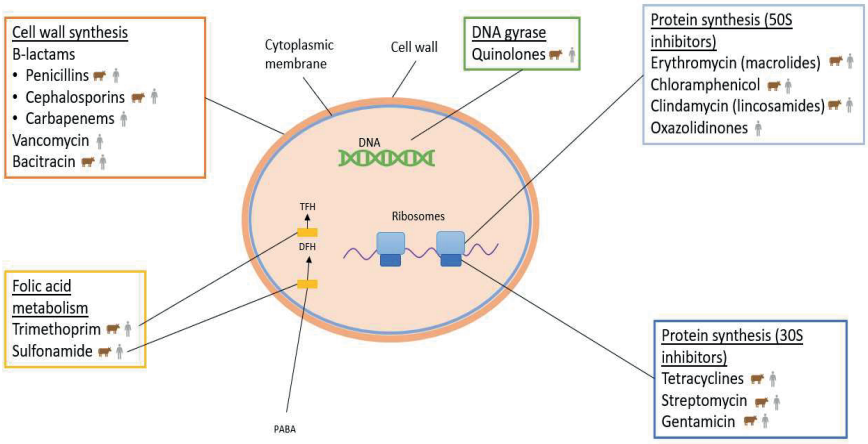


Figure 4. Common antimicrobial agents in veterinary and human medicine and modes of action. Use in veterinary and human medicine are indicated with an animal or human figure.

Resistance to the β-lactam penicillin can be mediated by the enzyme penicillinase, encoded by the *blaZ* gene, which hydrolyzes the β-lactam ring of penicillin and inactivates the drug. Resistance to penicillin and most other β-lactam antimicrobials can also be mediated by the penicillin binding protein PBP2a, encoded by *mec* genes (Peacock and Paterson, 2015), this resistance mechanism is discussed in more detail below. Resistance to tetracycline, a translation inhibitor used in both human and veterinary medicine, is often mediated by *tet* genes and in staphylococci of animal origin the most common are *tetK* and *tetL*, coding for membrane associated efflux proteins of the major facilitator superfamily, as well as *tetM* coding for a ribosome-protective protein (Wendlandt et al., 2013; Schwarz et al., 2018). The *tetK* and *tetL* genes are commonly found on plasmids, while *tetM* is often located on transposons (Schwarz et al., 2018).

Table 3. Overview of common antimicrobial resistance genes in staphylococci.

Gene	Antimicrobial agent	Properties
***mec* genes**	β-lactam anitmicrobials	Resistance through target replacement. Encodes additional transpeptidase penicillin binding protein (PBP), PBP2a. Located on staphylococcal cassette chromosome *mec* (SCC*mec*)
blaZ	Penicillins	Resistance through enzymatic drug-inactivation. Encodes the enzyme penicillinase Located on plasmid or chromosome
tetK ***tetL***	Tetracyclines	Encodes membrane associated efflux proteins. Often found on plasmids
tetM	Tetracyclines	Resistance through ribosomal protection. Encodes ribosome-protective protein. Commonly located on transposons
aacA-aphD ***aadD*** ***str***	Aminoglycosides	Resistance through enzymatic drug-inactivation. Encodes drug-inactivating enzymes. *aacA-aphD* located on transposon, *aadD* associated with plasmid.
***erm* genes**	Macrolides, lincosamides and streptogramins	Resistance through target modification, inhibiting drug-binding. Encodes methylases. Often found on mobile genetic elements (plasmids, transposons and others)
msrA	Macrolides and streptogramin B	Resistance through ribosomal protection. Encodes an ABC-F protein. Can be found on plasmids
mphC	Macrolides	Resistance through enzymatic drug-inactivation. Encodes macrolide phosphotransferase. Can be found on plasmids
lnuA	Lincosamides	Resistance via enzymatic drug-inactivation. Encodes nucleotidyltransferase. Commonly found on plasmids
vga	Streptogramin B (lincosamides)	Resistance through ribosomal protection. Encodes ribosomal protection protein
***dfr* genes**	Trimethoprim	Mediates resistance through target replacement. Encodes trimethoprim-resistant dihydrofolate reductases
fexA	Phenicols	Resistance via efflux. Encodes FexA protein of the major facilitator superfamily
***cat* genes**	Chloramphenicol	Resistance via enzymatic drug-inactivation
cfr	Phenicols and oxazolidinones	Resistance via target modification. Encodes the rRNA methylase Cfr. Have been detected on plasmids
optrA	Phenicols and oxazolidinones	Resistance via ribosome protection. Encodes an ABC-F protein Have been detected on plasmids

Aminoglycosides is another group of protein synthesis inhibitors, widely used for both humans and animals, but resistance mechanisms can target these agents as well. These mechanisms are based on several inactivating enzymes that differ in the spectra of substances they inactivate (Ramirez and Tolmasky, 2010). The *aacA-aphD* gene, located on a transposon, is widespread in staphylococci of animal origin and codes for a bifunctional enzyme conferring resistance to gentamicin, kanamycin and tobramycin (Rouch et al., 1987).

Several other aminoglycoside resistance gene are found in staphylococci, such as the *aadD* gene, coding for an adenyltransferase, conferring resistance to kanamycin, neomycin and tobramycin and the *str* gene conferring streptomycin resistance. The *aadD* gene was originally discovered on a plasmid that often integrates on SCC*mec* type II elements (Schwarz et al., 2018). Moving further through the range of different antimicrobial agents, resistance to macrolides, lincosamides and streptogramins, all agents that are used in both human and veterinary medicine, are also common in staphylococci. The resistance to these compounds is based on a diversity of genes coding for various resistance mechanisms. Of these, the most common are the *erm* genes, that confer combined resistance to both macrolides, lincosamides and streptogramins (Fessler et al., 2018), by coding for methylases that modify the targets and inhibit the drug from binding to the ribosome (Schwarz et al., 2018).

Altogether 14 different *erm* genes have been found in staphylococci of different sources (Feßler et al., 2018). These genes are often found on mobile genetic elements, both plasmids, transposons and others, allowing for horizontal spread of these genes (Feßler et al., 2018). While *erm* genes confer resistance to macrolides, lincosamides and streptogramins, the *msrA* gene, coding for a ribosomal protection protein (Sharkey et al., 2016), mediates resistance to macrolides and streptogramin B, and the *mphC* gene and *lnu* genes confer resistance to macrolides and lincosamides, respectively (Schwarz et al., 2018). The *vga* genes, also coding for a ribosomal protection protein, confer resistance to streptogramin A and, in some cases, lincosamides (Fessler et al., 2018).

Another compound used for both animals and humans is trimethoprim, an agent that inhibits synthesis of tetrahydrofolate, a precursor for DNA synthesis. Resistance to trimethoprim is based on the *dfr* genes (A, D, G and K), mediating resistance through target replacement by coding for trimethoprim-resistant dihydrofolate reductases. The *dfrA* and *dfrD* genes are most common staphylococci isolated from humans, while *dfrG* and *dfrK* have been found in staphylococci from several animal sources (Schwarz et al., 2018).

Resistance to phenicols can be mediated by different mechanisms. These include enzymatic inactivation via chloramphenicol acetyltransferases (encoded by *cat* genes), active efflux via exporters of the major facilitator superfamily (encoded by *fexA*), target site modification via the rRNA methylase Cfr or ribosome protection by the ABC-F protein OptrA (Schwarz et al., 2004; Schwarz et al., 2018).

In addition to these antimicrobial agents, used in both human and veterinary medicine, there are examples staphylococci of animal origin have been found to contain genes conferring resistance to drugs not approved for food-producing animals. Oxazolidinones, last-resort antimicrobials in human medicine, is an example (Cuny et al., 2017; Kang et al., 2020). These protein synthesis inhibitory agents are mostly not approved for veterinary use, but may be used for non-food producing animals in some countries. Two oxazolidinone resistance genes, *cfr* and *optrA,* are known to occur in staphylococci of animal origin (Li et al., 2016). These genes also confer resistance to phenicols, and both genes have been detected on plasmids (Schwarz et al., 2018), which could allow for their dissemination.

There are also several resistance determinants in staphylococci that are more common in human-associated strains. Vancomycin, a glycopeptide antibiotic that inhibits peptidoglycan crosslinking by binding to terminal alanine residues of the pentapeptides, is an important example. This antibiotic is commonly used to treat infections with multiresistant MRSA in hospitals. Enterococci can develop resistance to vancomycin, and the *van* genes conferring the resistance by altering the pentapeptide, are located on mobile genetic elements that have sporadically

appeared in MRSA (Foster, 2017), adding further complication to treatment of severe MRSA infections.

1.5. β-lactams

In the 1920s Alexander Fleming discovered the β-lactam penicillin while studying staphylococci, due to mold contamination of agar plates (Fleming, 1929; Kong et al., 2010). Today β-lactam antibiotics are the most commonly used antimicrobial class in medicine (Bush and Bradford, 2016). This applies to both human and veterinary medicine in Norway, where in the last decade β-lactams, and mainly β-lactamase sensitive penicillins, have been the most sold antimicrobial agents for both food producing terrestrial animals, companion animals and humans (NORM/NORM-VET, 2019). The widespread use of β-lactams is related to their low toxicity, good pharmacokinetics, oral availability and bactericidal action (Foster, 2019). These antimicrobials all feature a cyclic amide, known as the β-lactam ring (Fernandes et al., 2013), and hinder bacterial cell-wall synthesis (Peacock and Paterson, 2015).

Bacteria rely on an intact cell wall to maintain cell integrity and shape. The Gram-positive staphylococci, as the vast majority of other bacterial species, have a set of penicillin-binding proteins (PBPs) (Georgopapadakou and Liu, 1980) that are essential for the synthesis of peptidoglycan in the bacterial cell-wall. The peptidoglycan layer of the bacterial cell-wall is composed of glycan chains with alternating N-actylglucosamine (NAG) and N-acetylmuramic acid (NAM)-pentapeptide units that are cross-linked by peptide bridges. In staphylococci, the cross-links consist of five glycine residues and links the peptide side chains of adjacent glycan chains, resulting in the formation of a mesh-like structure of glycans and peptides.

Peptidoglycan synthesis requires two enzymatic processes. First, NAGs and NAMs are added to the peptidoglycan chain by transglycosylases while cross-links are created by transpeptidases (Typas et al., 2011; Egan et al., 2017). While transglycosylation can be performed both by PBPs and another group of

proteins known as the SEDS family (for sporulation, elongation, division and sporulation) (Meeske et al., 2016), only PBPs can carry out transpeptidation. In *S. aureus*, PBP1 and PBP3 are sole transpeptidases, while PBP2 can perform both transglycosylation and transpeptidation. The bactericidal effect of β-lactams is a result of inhibition of the transpeptidase activity of the PBPs (Peacock and Paterson, 2015; Bush and Bradford, 2016). β-lactams act as substrate analogs of the pentapeptide side chain and bind covalently to the transpeptidase active site serine of PBPs to inhibit the enzymatic activity (Chambers, 1997; Peacock and Paterson, 2015) eventually leading to weakened cell wall and cell-death.

There are several different classes of β-lactams, such as penicillins, cephalosporins and carbapenems. The first clinically used β-lactam was the penicillinase-sensitive penicillin G (benzylpenicillin), used to treat streptococcal and staphylococcal infections (Rammelkamp and Keefer, 1943). In Norway, this β-lactam is still the drug of choice in terrestrial production animals, including for bovine mastitis, according to the national therapy recommendations (Norwegian Medicines Agency, 2012). Phenoxymethylpenicillin is another penicillinase-sensitive penicillin that is still widely used in human medicine, for instance for children (Pottegård et al., 2015). However, quickly after the introduction of penicillin in the 1940s, resistant penicillinase-producing staphylococci emerged and spread. These strains have a penicillinase, a β-lactamase enzyme that hydrolyze the β-lactam ring and inactivate the drug (Kirby, 1944). This led to the development of penicillinase-stable penicillins, such as methicillin, oxacillin, cloxacillin and nafcillin, that were all used until the emergence of MRSA in the late 1970s (Bush and Bradford, 2016). In the 1970s penicillins with increased effect against Gram-negatives, such as ampicillin and amoxicillin, were also introduced (Bush and Bradford, 2016), and amoxicillin is still widely used for companion animals in Norway, both alone or in combination with a β-lactamase inhibitor (NORM/NORM-VET, 2019). Betalactamase inhibitors are agents that bind to the active site of the betalactamases, inactivating the enzyme (Drawz and Bonomo, 2010).

Shortly after the advance of penicillinase-stable penicillins, in the 1950s, the discovery of the naturally occurring penicillinase-stable β-lactam cephalosporin C, led to the development of a range of new cephalosporins (Newton and Abraham, 1956; Abraham, 1987). These agents have since the 1970s been some of the most potent and widely used antimicrobials (Fernandes et al., 2013). Cephalosporins are often separated into first to fifth generation by their antibacterial spectrum, differing also in β-lactamase stability, absorption, metabolism, stability and side effects. The first generation cephalosporins are very effective against Gram-positive cocci and moderately active against some Gram-negative rods, while fifth generation cephalosporins were developed to target resistant bacteria and can be effective against MRSA (Fernandes et al., 2013).

Of the carbapenems, imipenem was the first developed as a therapeutic agent used to treat infections caused by Gram-positive, Gram-negative and anaerobic microorganisms (Bush and Bradford, 2016). These substances are known for their broad spectrum and stability against most β-lactamases (Bonfiglio et al., 2002). However, there has been an emergence of carbapenemases in Gram-negative bacteria, that may inactivate all β-lactam antimicrobials (Bush, 2013).

Several of the β-lactam antimicrobials are considered critically important due to their significance in human medicine and the non-human use of the same antimicrobials, making them a priority in the efforts to preserve the effectiveness of the antimicrobials. WHO lists cephalosporins of third generation and higher as critically important antimicrobials due to their significant role in human medicine and widespread non-human use. Carbapenems are also listed as critically important for human use, while penicillins and cephalosporins of first and second generation is listed as highly important (WHO, 2019).

1.6. Methicillin resistance

Methicillin is a semi-synthetic penicillinase-stable β-lactam antibiotic. Shortly after the introduction of this agent in 1959, reports of methicillin resistant isolates of *S. aureus* and coagulase-negative staphylococci emerged. Methicillin is no longer

used or produced commercially, but the term methicillin resistance persists and incorporates resistance to almost all β-lactam antibiotics except fifth generation cephalosporins (Peacock et al 2015).

1.6.1. Genetic basis and mechanism

The *mec* genes are responsible for encoding the resistant phenotype of methicillin resistant staphylococci. There are several *mec* genes found in staphylococci, where *mecA* is the most studied. In addition, *mecC* and recently *mecB* have been found in *S. aureus* (Paterson et al., 2014; Becker et al., 2018). These *mec* genes encode an additional transpeptidase PBP, known as PBP2a, with a low affinity for β-lactam antimicrobials (Partridge et al., 2018). PBP2a enables resistant strains to continue cell-wall synthesis even in the presence of β-lactams, as the enzyme continues the transpeptidase activity when other PBPs are inhibited (Peacock and Paterson, 2015). Methicillin-susceptible *S. aureus* does not have an equivalent of the *mec* genes found in MRSA (Chambers, 1997). The *mecA* and *mecC* genes, are located on the mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) that is inserted into the staphylococcal chromosome (Becker et al., 2014a), while *mecB* has recently been found on a plasmid in *S. aureus* (Becker et al., 2018).

Several factors can affect the expression of *mecA*. Most importantly a signal transduction system encoded by regulatory genes *mecR1* and *mecI*, located close to *mecA* on the SCC*mec* which is activated in response to B-lactam exposure. This system is homologous to the regulatory system of betalactamase (*blaZ*) expression in *S. aureus* (the *blaR1-blaI* system), with the *bla*-system also being able to affect the expression of *mecA* in strains harboring both systems. In addition, both environmental factors and stress can influence the expression, such as the reversal of induction of resistance in the absence of antibiotics and the heterogenous expression of resistance seen in most MRSA isolates (Peacock and Paterson, 2015).

The SCC*mec* elements are diverse in both structure and genetic content (IWG-SCC, 2009) and can also carry other resistance determinants and virulence properties (Rolo et al., 2017). It consists of two important units, the *mec* complex,

composed of *mecA* (or *mecC*) and the two regulatory genes (*mecR1* and *mecI*) and a *ccr* complex, containing cassette chromosome recombinase genes (*ccr*), that are responsible for the movement of the cassette (IWG-SCC, 2009; Kim et al., 2012). In addition, the SCC*mec* consists of so-called joining regions (J1 to J3) that may contain IS-elements, transposons and plasmids, including genes encoding a variation of function including resistance to other antibiotics and heavy metals (Rolo et al., 2017). There is evidence indicating that the evolution of SCC*mec* started in NAS species, more specifically the *Staphylococcus sciuri* group (Rolo et al., 2017). The *mecA1* gene, found ubiquitously in *S. sciuri* with 80 % nucleotide identity with *mecA*, is believed to be the most likely evolutionary origin of *mecA* in MRSA (Couto et al., 1996; Wu et al., 1996). Homologues closer to *mecA* have also been found in other NAS species, such as *mecA2* in *Staphylococcus vitulinus* with 90 % identity and *mecA* in *Staphylococcus fleurettii* with 99 % identity (Schnellmann et al., 2006; Tsubakishita et al., 2010b) to the *mecA* found in *S. aureus*. In *S. fleurettii* the *mecA* gene is also found in connection with the two regulator genes (*mecR1* and *mecI*), indicating that the assembly of the *mec* complex might have occurred in this species (Tsubakishita et al., 2010b). There are also indications that *S. sciuri* is the evolutionary source of the recombinase genes (*ccr* complex), also associated with the assembly of SCC*mec* (Rolo et al., 2017).
In contrast to *mecA* and *mecC* that are located on SCC*mec*, the *mecB* gene has recently been detected on a plasmid in a MRSA strain (Becker et al., 2018). This gene has previously been found in *Macrococcus caseolyticus*, a common colonizer of animal skin, both as part of an SCC*mec* element on the chromosome and on a plasmid (Baba et al., 2009; Tsubakishita et al., 2010a). Becker et al. (2018) showed that the *mecB* carrying plasmid in *S. aureus* UKM4229 was distantly related to a macrococcal plasmid, indicating a possible transfer of genes between the two genera. In addition to *mecA*, *mecB* and *mecC*, a *mecD* genes have been discovered in bovine and canine *M. caseolyticus* isolates (Schwendener et al., 2017).

1.7. Methods for detection of resistance and virulence in staphylococci

There are several methods for studying resistance and virulence, both phenotypic and genotypic. The most common methods for studying phenotypic antimicrobial susceptibility are the disk diffusion methodology and minimal inhibitory concentration (MIC). Disk diffusion is based on measurement of inhibition zone diameters around antimicrobial disks on agar plates, where the inhibition zone is the area where no visible growth is detected and the size of the zone indicates whether the bacteria are resistant against the agent or not (Matuschek et al., 2014). MIC on the other hand is defined as the lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism (Andrews, 2001)

Disk diffusion is one of the oldest methods for testing antimicrobial susceptibility and is still widely used in routine microbiological laboratories. The widespread use of the method is probably due to the great versatility; the method can be used for the majority of bacterial pathogens and almost all antimicrobial agents and requires no advanced equipment (Matuschek et al., 2014). The results of the test, deeming the bacteria as susceptible or resistant, are also easy to understand and use in guiding therapy. MIC is often used in research as a tool to determine antimicrobial susceptibility due to requiring some equipment such as a MIC panel, although it is used in routine laboratories as well, mainly to confirm resistance (Andrews, 2001). Both methods require careful adherence to the methodology in order to give reliable results, including accurate preparation of standardized inoculum, inoculation of the bacterial suspension, incubation and reading of agar plates or MIC panel. The adherence to methodology is important both in terms of execution and time (Bayot and Bragg, 2020).

In addition to the importance of correct procedure, the breakpoints used to classify a microorganism as susceptible or resistant are central to the interpretation of the results. The epidemiological cut-off value (ECOFF) is the smallest inhibition zone diameter or highest MIC for microorganisms devoid of any phenotypically detectable acquired resistance mechanisms for a given substance and species.

However, the ECOFF does not predict clinical susceptibility (EUCAST, 2018). The clinical breakpoints are related to treatment and defines a microorganism as susceptible, intermediate, or resistant. These breakpoints are based on a range of data, such as disease indication, clinical response, dosing, pharmacokinetics and pharmacodynamics, safety and various microbiological data and are not necessarily able to distinguish between bacteria with and without acquired resistance mechanisms, but used to guide antimicrobial therapy (Kahlmeter et al., 2003). The ECOFF on the other hand, is based on zone diameters or MIC distributions in the wild-type population of a given species, making it possible to detect acquired resistance mechanisms. A graphical presentation of MIC distribution and ECOFF for *S. aureus* and cefoxitin is shown in Figure 5. The ECOFFs are particularly important to ensure that clinical breakpoints do not divide wild-type populations (Kahlmeter et al., 2003).

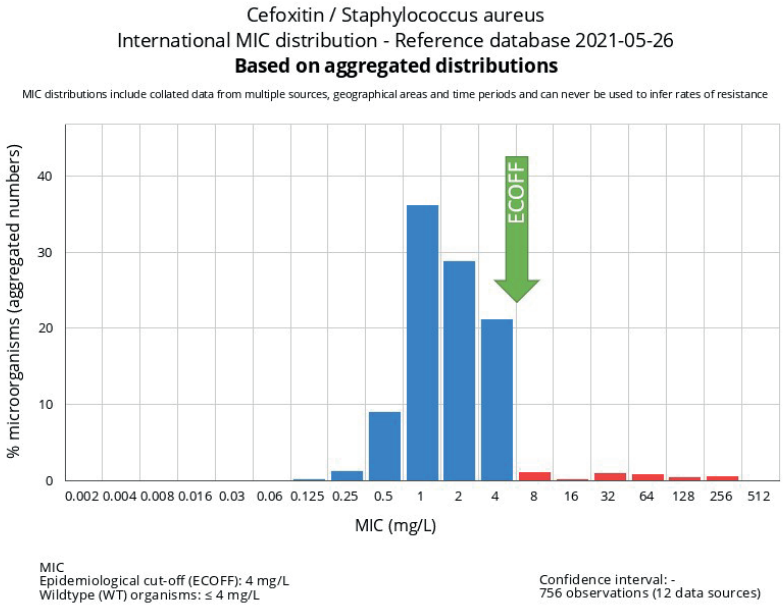


Figure 5. MIC distributions and ECOFF for *S. aureus* and cefoxitin (wildtype shown in blue). These data have been produced in part under ECDC service contracts and made available by EUCAST at no cost to the user and can be accessed on the EUCAST website www.eucast.org. EUCAST recommendations are frequently updated and the latest versions are available at www.eucast.org.

In addition, to be able to compare results of phenotypic antimicrobial susceptibility testing, there is a need for standardized methodology and clinical breakpoints between laboratories and countries. Previously there was no standardized common method or breakpoints for Europe, but EUCAST has harmonized the European MIC breakpoints (Kahlmeter et al., 2003) and developed a standardized disk diffusion methodology, as well as zone diameter breakpoints (Matuschek et al., 2014).

There are different approaches to the study of virulence in staphylococci by phenotypical methods. Some characteristics, such as ability to produce coagulase and hemolysins, are directly associated with virulence and are also utilized for diagnostic purposes (Wiseman, 1975; Peetermans et al., 2015). Other properties, such as the capacity of biofilm formation, are, as described above, involved in the pathogenesis of both *S. aureus* and NAS, and may be used as one indicator of virulence.

Besides analyses for phenotypic resistance and virulence, there are several ways of studying the genetic basis for these bacterial traits. Polymerase chain reaction (PCR), one of the basic methods in molecular biology, is an extensively used method for searching for specific sequences in the bacterial DNA, such as antimicrobial resistance or virulence genes. Based on repeated cycles of DNA synthesis directed by oligonucleotides and primers, the method performs *in vitro* replication to amplify and detect target DNA sequences (Persing, 1991). Although the method is widely used, it also has pitfalls. The method will only detect the specific target sequence and is unable to detect new sequences with similar properties.

Another way of investigating the genetic background for resistance and virulence is by use of WGS which can detect the presence of both antimicrobial resistance and virulence genes, from specific genes to determining the total resistome or virulome (Rossen et al., 2018). One of the most important and interesting aspect regarding application of WGS results for understanding of antimicrobial resistance and virulence is the possibility of predicting clinical resistance and virulence phenotypes from the data. The presence of SCC*mec* in

S. aureus, for example, is a reliable predictor of β-lactam resistance (Balloux et al., 2018).

Predicting the full resistance profile is possible in some cases, but the concordance between WGS results and phenotypic AMR varies between bacterial species. The relationship between chromosomal mutations and the related phenotypic changes in *Enterobacteriaceae*, for example, is not always well characterized and WGS ability to predict resistance is likely to be restricted by existing knowledge about factors contributing to resistant phenotypes (Ellington et al., 2017). In *S. aureus,* however, the problem of antimicrobial resistance has driven extensive studies to identify the genetic basis of resistance that have revealed a spectrum of mechanisms, which in turn has proven to be a valuable resource for prediction of antimicrobial resistance by WGS (Ellington et al., 2017). In fact, several studies have shown a good concordance between phenotypic antimicrobial resistance and WGS-based prediction in *S. aureus* (Holden et al., 2013; Gordon et al., 2014; Bradley et al., 2015). Even so, most predictions of antimicrobial resistance based on WGS data are qualitative, predicting only if an isolate is expected to be resistant or susceptible (Balloux et al., 2018), while clinical resistance can be affected by several other factors, such as the genetic background of the strain (Luo et al., 2005), regulatory systems and modulating effects by the environment (Baym et al., 2016).

In terms of virulence, there are some tools available for determining virulence profiles (Joensen et al., 2014). However, virulence is context-dependent and often a complex interplay between microorganisms and host. Thus, although it is possible to predict potential virulence profiles using WGS, it is uncertain how well this correlates with the actual virulence expression of the isolate and pathogenic potential during infection (Balloux et al., 2018; Naushad et al., 2019). Both decreasing costs and higher accessibility have made WGS an increasingly used tool within microbiology. However, there are several complex issues that needs to be studied further.

2. Aims of study

This study was part of a larger, international Animal Health and Welfare (ANIHWA) ERA-NET project with the title "Bacteriophages as alternative to antimicrobial treatments of bovine mastitis caused by methicillin-resistant staphylococci (MRS), with emphasis on methicillin-resistant *Staphylococcus aureus*". The responsibility of the NMBU-group in this project, and thus the overall aim of my PhD project, was to increase the knowledge on antimicrobial resistance and virulence in bovine mastitis-related staphylococci, and to examine the mechanisms related to methicillin resistance. The overall aim of this thesis was accomplished though the following objectives:

1. Study of the interplay between PBP2a and different β-lactam antimicrobials to gain more insights into how PBP2a mediates β-lactam resistance in *S. aureus* (Paper I).
2. Examine the presence of antimicrobial resistance and virulence characteristics of three groups of *S. aureus* and NAS isolates from bovine milk samples from Norway and Belgium collected as part of the project (Paper II).
3. Analyze antimicrobial resistance and virulence genes in staphylococci, associated with mastitis in dairy cattle, by using whole genome sequencing (WGS) to study the content of genes related to antimicrobial resistance and virulence (Paper III).

3. Summary of papers

Paper I: Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220

Marte Ekeland Fergestad, Gro Anita Stamsås, Danae Morales Angeles, Zhian Salehian, Yngvild Wasteson and Morten Kjos

MicrobiologyOpen 2020, doi: 10.1002/mbo3.1057

Resistance to β-lactams in MRSA strains is mediated by the *mecA*-encoded PBP2a protein. The aim of this study was to gain more insights into how PPB2a protects staphylococci against different β-lactams. Expression of *mecA* is affected by different factors. To allow controlled expression of PBP2a in *S. aureus*, we therefore introduced an IPTG-inducible *mecA* into the methicillin sensitive laboratory *S. aureus* strain RN4220. We confirmed, through growth assays and single-cell microfluidics time-lapse microscopy experiments, that PBP2a expression protects against β-lactam antimicrobials. In the growth assays we tested a panel of nine different β-lactams, which made us able to conclude that there is also a large variation in the level of protection conferred by PBP2a towards different agents. Expression of PBP2a resulted in an only fourfold increase in minimum inhibitory concentration (MIC) for imipenem, while a 32-fold increase in MIC was observed for cefaclor and cephalexin. In our experimental setup, PBP2a conferred the highest protection against cefaclor and cephalexin; two β-lactams that are known to have a high specific affinity toward the transpeptidase PBP3 of *S. aureus*. By using a single-cell microfluidics setup we could demonstrate a substantial phenotypic variation between cells when exposed to β-lactams and show that *mecA*-expressing *S. aureus* can survive β-lactam concentrations considerably higher that the minimal inhibitory concentration.

Paper II: Antimicrobial resistance and virulence characteristics in three collections of staphylococci from bovine milk samples

Marte Ekeland Fergestad, Anneleen De Visscher, Trine L'Abee-Lund, Cyrille Ngassam Tchamba, Jacques G. Mainil, Damien Thiry, Sarne De Vliegher and Yngvild Wasteson

Journal of Dairy Science 2021, doi: 10.3168/jds.2020-19988.

The aim of this study was to identify and characterize three collections of staphylococcal isolates from bovine milk samples with respect to antimicrobial resistance and virulence, with emphasis on methicillin resistance and virulence genes typically displayed by *S. aureus*. A total of 272 staphylococcal isolates collected in Norway and Belgium in 2016 with the following distribution were included: Group 1, Norway, 100 isolates; Group 2, Flanders, Belgium, 64 isolates; Group 3, Wallonia, Belgium, 108 isolates. The isolates were species identified by Maldi-tof MS, phenotypic resistance was determined by disk diffusion and PCR was performed for detection of methicillin resistance genes *mecA* and *mecC* and selected virulence genes. Of the total of 272 isolates there were 45 *S. aureus* isolates and 227 NAS isolates from 16 different species. Resistance to trimethoprim-sulfonamide was most frequently observed in Group 2 and 3, while in Group 1 resistance to penicillin was most common. In all three groups antimicrobial resistance was common in *S. epidermidis* and *S. haemolyticus*, with resistance to trimethoprim-sulfonamide frequently occurring in both species, as well as *S. aureus*. Ten Belgian isolates (one from Group 2, nine from Group 3) carried the methicillin resistance determinant *mecA*: five *S. aureus* from two different farms and five NAS from three different farms. Almost all *S. aureus* isolates were positive for at least three of the screened virulence genes, while in total, only eight NAS isolates harbored any of the same genes. The virulence genes *clfA* and *fnbpA* were identified in over 80 % of *S. aureus* isolates in all three groups, *hlb* were identified in the majority of isolates in Group 1 and 3, and in over half of the isolates in Group 2. The toxic shock syndrome toxin gene *tsst01* and fibronectin binding protein gene *fnbpB* were the least frequently detected virulence genes in *S. aureus*.

Paper III: Whole Genome Sequencing of Staphylococci Isolated from Bovine Milk Samples in Belgium and Norway

Marte Ekeland Fergestad, Fabrice Touzain, Sarne De Vliegher, Anneleen De Visscher, Damien Thiry, Cyrille Ngassam Tchamba, Jacques G. Mainil, Trine L'Abee-Lund, Yannick Blanchard and Yngvild Wasteson

Submitted Frontiers in Microbiology, May 2021

We used Illumina sequencing to whole genome sequence 93 staphylococcal isolates selected from the collection of isolates in paper II, 45 *S. aureus* isolates and 48 NAS isolates. All NAS species identified in paper II were included in the selection. We determined the content of antimicrobial resistance genes and virulence genes in the 93 staphylococcal isolates. Several antimicrobial resistance genes were frequently observed in different NAS species, especially in species from clade D (*S. devriesei, S. epidermidis, S. haemolyticus, S. hominis* and *S. warneri*). The lincosamide resistance gene *lnuA* and penicillin resistance gene *blaZ* were most frequently identified and were present in several NAS species, as well as *S. aureus*. The *erm* genes conferring macrolide resistance were also identified in several NAS isolates and a small number of *S. aureus* isolates. In most *S. aureus* isolates, no antimicrobial resistance genes were detected, but in five *S. aureus* isolates three to six resistance genes were identified and all five of these carried the *mecA* gene. As for the content of virulence genes, these were more frequently identified in *S. aureus*, compared to NAS. Using VirulenceFinder we detected several virulence genes in *S. aureus*, but very few in NAS, while by using the database compiled by Naushad et al. (2019) we detected several virulence genes in both *S. aureus* and NAS. Most NAS species carried on average 26 virulence genes, however, *S. chromogenes* and *S. hyicus* (clade B) stood out from the rest with 44 and 98 virulence genes respectively, although *S. hyicus* was only represented by one isolate. *S. aureus* isolates had on average 140 virulence genes.

4. Discussion

4.1. Methodological considerations

4.1.1. Materials

The *S. aureus* strain RN4220 was used for the study of the interplay between heterologous expression of methicillin resistance and the different β-lactam antimicrobials (paper I). Clinical isolates of *S. aureus* are mostly difficult to transform, due to restriction-modification systems that prevents uptake of foreign DNA (Corvaglia et al., 2010). Unlike clinical strains, the RN4220 is a commonly used laboratory strain harboring mutations causing it to be restriction deficient, thus making it an ideal cloning host. However, the strain has also accumulated other mutations that could affect the fitness and general behavior of the strain (Nair et al., 2011). These factors separate it from clinical strains and could influence the results. Nevertheless, *S. aureus* RN4220 and related strains are frequently used as model strains for various studies, including antibiotic resistance (Nair et al., 2011; Ballhausen et al., 2014), and we therefore chose to it as host strain for heterologous expression of *mecA* in this work.

The collection of samples for paper II and III were collected from three geographical locations: Norway, Flanders (Belgium) and Wallonia (Belgium), as part of a larger European collaboration. In Norway, staphylococci were isolated from samples submitted to the routine diagnostics of the TINE Norwegian Dairies Mastitis Laboratory, samples came from 100 herds and one quarter milk sample was collected from each herd. The *S. aureus* samples were isolated in pure culture from clinical mastitis milk samples, while the NAS samples were isolated from milk samples with rich growth of NAS in pure culture, submitted to the laboratory due to high SCC or clinical mastitis. In Flanders, all quarters from lactating cows from two herds were sampled during one cross-sectional sampling, but as *S. aureus* were not isolated in milk samples from the cross sectional sampling, the *S. aureus*

isolates were collected from an additional collection from eight herds (De Visscher et al., 2016). In Wallonia three herds with a subclinical mastitis problem were selected for sampling. All cows with a cow somatic cell count (cSCC) > 300 000 cell/ml on the last DHI-sampling were selected (n=114) and all quarters were sampled.

The differences in sampling procedures were conveniently adapted to the national compositions of the dairy industry, as well as the framework, ambitions, and resources of the collaborative project. These three collections ensured diversity of the material, however, this also meant that a comparison between the three collections and the three geographical regions was not possible. Because of this the samples were separated into three distinct collections for paper II; Group 1 consisted of isolates from Norway, Group 2 of isolates from Flanders, Belgium and Group 3 consisted of isolates from Wallonia, Belgium.

For paper III samples were selected from the collection from paper II with the following criteria: all *S. aureus* isolates were included and all NAS species should be represented. The NAS isolates were further selected based on phenotypic antimicrobial resistance patterns.

4.1.2. Expression studies

Expression of *mecA* in MRSA-strains is in most strains regulated by the *mec*-regulatory system, but the transcription is also influenced by a diversity of other genetic factors (including other regulatory systems) and environmental factors (Peacock and Paterson, 2015). Therefore, to be able to control expression of *mecA* in *S. aureus*, we introduced the gene downstream of the P_{spac} promotor on the low-copy number plasmid pLOW in the methicillin sensitive laboratory *S. aureus* strain RN4220, allowing for controlled expression of the *mecA* gene from the pLOW plasmid (Liew et al., 2011) by adding increasing concentrations of IPTG. Plasmids were initially constructed in the *Escherichia coli* strain IM08B (Monk et al., 2015); the pLOW-*mecA* plasmid was created by amplifying *mecA* by PCR from the *S. aureus* MRSA strain COL, digesting the PCR product with restriction

enzymes and ligating the product into the multiple cloning site of pLOW. The plasmid was then transformed into *S. aureus* RN4220 by electroporation (Löfblom et al., 2007).

In addition, we created two mutants by introducing a point mutation in the *mecA* gene using a two-step overlap extension PCR, one with a mutation in the active site (*mecA*(S403A)) and one with a mutation in the key residue for allosteric regulation of PBP2a activity (*mecA*(K188A)) (Otero et al., 2013). These were also introduced into the pLOW plasmid and transformed into *S. aureus* RN4220.

To determine the level of protection against different β-lactam antimicrobials, the cells were exposed to a panel of nine β-lactams consisting of the following: ampicillin, cefaclor, cefotaxime, cefoxitin, cephalexin, imipenem, oxacillin, penicillin G and piperacillin. In order to reveal any systematic differences between the β-lactams, they were chosen based on their class (the subset includes penicillins (1st–4th generation), carbapenems, and cephalosporins (1st–3rd generation)) and their reported specificity to different PBPs (cefotaxime with highest affinity for PBP2, cefoxitin for PBP4, and cephalexin and cefaclor for PBP3). The MICs were determined by twofold dilution assays in microtiter plates with various IPTG concentrations. The plates were incubated at 37°C and growth was monitored every 10 minutes for 15 hours by measuring OD_{600}.

We also performed microfluidics fluorescence time-lapse microscopy to further examine, on a single-cell level, how heterologous expression of *mecA* protected against β-lactams. *S. aureus* RN4220 with a functional *mecA* and a *S. aureus* RN4220 with a *mecA* harboring an active site mutation were exposed to concentrations of cefoxitin and cefotaxime twofold and 20-fold higher than MIC for *S. aureus* RN4220. The cells were grown for 6 hrs for cefoxitin and 4 hrs for cefotaxime. After cefotaxime exposure cells were switched to cefotaxime-free medium to study the potential recovery and regrowth of cells after antimicrobial exposure.

4.1.3. Phenotypic antimicrobial susceptibility testing

The disk diffusion method by Bauer et al. was used to determine the phenotypic antimicrobial susceptibility of the isolates to a panel of antimicrobials. The method was performed according to the methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.com, accessed august 2018), except Mueller Hinton broth was used instead of saline water to prepare the bacterial suspension corresponding to 0.5 McFarland.

The tested panel consisted of the following antimicrobials: ampicillin (10 µg), amoxicillin and clavulanic acid (20+10 µg), ciprofloxacin (5 µg), clindamycin (10 µg), erythromycin (15 µg), gentamicin (10 µg), linezolide (10 µg), penicillin (1 U), trimethoprim (5 µg), sulfonamide and trimethoprim (19:1, 25 µg), and tetracycline (30 µg). As recommended by EUCAST (www.eucast.com, accessed august 2018), cefoxitin was used for determination of phenotypic methicillin resistance. *S. aureus* ATCC 29213 was used as quality control. The isolates were categorized as resistant, intermediate (assigned as resistant) or susceptible based on clinical breakpoints determined by EUCAST. For NAS species, general breakpoints for coagulase negative staphylococci were used.

The disk diffusion method has been used for a long time and is still widely used in clinical microbiology laboratories. The technique was chosen because it requires no advanced equipment, is suitable for staphylococci and almost all antimicrobial agents can be tested. In addition, it is easy to adapt and incorporate different antimicrobial agents. The method is reproducible and accurate if done according to standards. There are, however, several factors that can affect the zone diameters. User-related factors such as inoculum density, age of colonies, incubation temperature and time and reading of zone diameters will cause variations in zone diameters, as well as external factors like variations between batches of media and disks (Matuschek et al., 2014).

The use of Mueller Hinton broth instead of saline water is not expected to have interfered with the results because the same medium (Mueller Hinton agar) is used in the agar plates for the disks of the panel of antimicrobial agents. According

to methodology, the suspension was used within approximately 15 minutes and always within 60 minutes, to ensure that there was no significant bacterial growth in the tube.

The isolates were categorized as resistant, intermediate (assigned as resistant) or susceptible based on clinical breakpoints determined by EUCAST. For NAS species, general breakpoints for coagulase negative staphylococci were used. Following the methodology and breakpoints determined by EUCAST should reduce the potential margins of error when performing the disk diffusion and interpreting the zone diameters. However, the EUCAST breakpoint tables, at the time of use in paper II, were mostly inferred from human, not animal strains, potentially affecting our results. In addition, using general breakpoints for NAS isolates might be misleading, as evidence show that this is not a uniform group and there are several differences between the species (Supré et al., 2011; Vanderhaeghen et al., 2014; Condas et al., 2017b).

4.1.4. Polymerase chain reaction

Polymerase chain reaction (PCR) was chosen to uncover virulence genes and selected antimicrobial resistance genes, as well as in several steps of constructing the pLOW*mecA* plasmid. The PCR for detection of *mecA* and *mecC* was performed according to the protocol by Stegger et al. (2012). For detection of virulence genes, nine genes commonly found in *S. aureus* isolates were selected based on a review of relevant articles available in the PubMed database. The PCRs for the chosen genes were performed according to Wang et al. (2016) for *clfA, fnbpA, fnbpB, hla, hlb* and *tsst01*, according to Salasia et al. (2004) for *ssl7* and Ote et al. (2011) for *cap5* and *cap8*. This method of detecting and amplifying target DNA sequences is widely used. However, since the method is dependent on target specificity, discovering new resistance mechanisms is not possible, and silent genes, or pseudo-genes can cause false positive results, while mutations or deviations in primer binding sites may generate false negative results.

Screening for many genes in a large collection of isolates can be time consuming because different genes need different primers with individual annealing temperatures. Similar genes in different bacterial species might require different primers, making screening of a large array of genes laborious. The primers selected for the detection of virulence genes in bacterial strains investigated in this study were inferred from virulence genes in *S. aureus*. This could potentially affect detection of virulence genes in NAS species. If these genes were present in NAS species with a deviating sequence in the primer-binding site, creating a mismatch between primer and target sequence, the PCR might not detect the genes. In addition, the method is not specific beyond that it detects a match to the primer sequences, meaning it could detect homologues as long as there is a match between primer and binding site, but not separate between the different homologues.

Five NAS species were positive for the *mecA* gene, but three of these were not phenotypically cefoxitin resistant. When these isolates were re-tested with a different set of primers for *mecA* and *mecC*, two isolates, one *S. xylosus* and one *S. sciuri,* turned out negative for the *mecA* gene. The WGS revealed that these three cefoxitin susceptible NAS isolates harboured *mecA* homologues, such as *mecA1* in *S. sciuri* and *mecC2* in the *S. xylosus*. However, the WGS also identified the *mecA1* gene in all *S. sciuri* isolates. The *mecA1* gene is thought to be ubiquitous in *S. sciuri* and does generally not confer methicillin resistance. The fact that one primer pair detected some of these *mec*-homologues, but not all and the other pair did not detect any shows the complexity of the detection of the *mec*-genes using PCR.

4.1.5. Whole genome sequencing

A selection of 95 staphylococcal isolates, selected from the original collection of 273 isolates, were whole genome sequenced. The genomic DNA libraries were prepared for Illumina sequencing according to the manufacturer's instructions using the Nextera XT kit and sequenced by the NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA). The sequencing, assembly, and preparation of WGS results was done at the French Agency for Food, Environmental and

Occupational Health and Safety (ANSES), during the ANIHWA ERA-NET project. In recent years rapidly declining costs and increase in quality of WGS have made this technology a reasonable tool for antimicrobial resistance research, diagnostics and surveillance, and using the method directly on clinical samples will reduce time frame compared to the traditional methods (Zankari et al., 2013). It is, nevertheless, essential with good quality samples and well curated databases to get good results.

Other factors to consider, at least when performing similarity searches, are the settings for resolution and the limits for identity, mismatches and gaps, and also the length of the sequence. Additionally, WGS will struggle to detect new resistance mechanisms and point mutations causing resistance that are not covered by databases (Ellington et al., 2017). Lastly, the presence of an antimicrobial resistance gene is not necessarily synonymous with expression of resistance (Zankari et al., 2013).

WGS does, however, make it possible to screen for several resistance genes at once and in *S. aureus*, where the genetic basis of antimicrobial resistance is well studied, WGS can be used to predict antimicrobial resistance (Ellington et al., 2017). This is the case with regard to detection of the *mec*-genes, WGS can be a useful tool to complement phenotypic methods, as the detection of SCC*mec* has been shown to correspond well with phenotypic methicillin resistance (Balloux et al., 2018). Many of the same issues regarding WGS and the detection of antimicrobial resistance applies for the detection of virulence genes as well. The need for good quality samples and curated databases are essential, and the difficulties of detecting new virulence mechanisms are important. Regarding virulence, the interaction between the microorganism and the host is central to pathogenicity. This is often a complex interplay where the association between the content of virulence genes of the infecting strain and the actual virulence in the patient is difficult to predict (Balloux et al., 2018; Naushad et al., 2019).

4.2. General discussion

The genus *Staphylococcus* consists of a range of different species. Because they can be found as commensals on skin and mucus membranes of humans and animals and are also capable of causing infection in a range of tissues and species, continuous knowledge about their characteristics is needed. In addition to their large infection spectrum, these bacteria can accumulate a wide array of antimicrobial resistance mechanisms, further emphasizing the need for updated knowledge.

This thesis sheds light on one of the most important resistance mechanisms in staphylococci, the methicillin resistance or β-lactam resistance, by studying the interaction between methicillin resistant *S. aureus* and different β-lactam antimicrobials. The work revealed some interesting findings regarding resistance against β-lactams targeting different PBPs that should be further investigated. It has also highlighted important considerations connected to the treatment of MRSA infections. Further, this thesis contributes with knowledge to the ever-changing pattern of antimicrobial resistance and virulence in bovine staphylococci, both on the well-studied *S. aureus*, but also NAS, whose characteristics and significance remain less clear. Such information is needed for adapting antimicrobial use and to ensure the ideal treatment of staphylococcal infections, both in human and veterinary medicine for the benefit of human and animal health as well as food safety for consumers.

In paper I the interplay between methicillin resistant *S. aureus* and different β-lactam antimicrobials were examined. Paper II describes the phenotypic antimicrobial resistance and genotypic virulence detected by PCR of staphylococci from bovine milk samples. Paper III describes the genotypic virulence and resistance of a selection of staphylococci from bovine milk samples by use of whole genome sequencing. In the following sections, results are discussed in a broader context.

4.2.1. PBP2a provides variable protection toward different β-lactams in *S. aureus* RN4220

By introducing an IPTG-inducible *mecA* gene into the methicillin sensitive *S. aureus* strain RN4220 we initially confirmed that heterologous expression of PBP2a provides protection towards the β-lactam cefoxitin. We also established that a mutation in the active site of PBP2a (*mecA*(S403A) which as expected completely abolished the resistance. However, a mutation in the allosteric site (*mecA*(K188A) did not show any differences in protection levels compared to the *mecA* expressing strain, suggesting that allosteric regulation of PBP2a did not play a key role in our experimental setup.

We noted that he level of resistance towards cefoxitin was relatively low upon heterologous expression compared to MRSA strains carrying the full SCC*mec* cassette. It is possible that the different strains have variable expression of PBP2a. We attempted to use Western blotting with PBP2a-specific antibodies to monitor the relative expression of PBP2a in our modified strains compared to MRSA strains, however, this was not successful to poor specificity of the antibody. In addition to the expression level, there might be unknown strain-dependent factors at play. It is probable that the PBP2a functions more optimally in the natural MRSA host compared to the susceptible laboratory strain RN4220, due to function being optimized to the natural host (Katayama et al., 2003). For example, it is possible that optimal function is dependent on the full SCC*mec* cassette. In this context, it should also be noted that the horizontal gene transfer observed for *mecA* in *S. aureus* is relatively limited (Peacock and Paterson, 2015). It could be speculated that this may be due to a relatively low resistance level in strains with recently acquired *mecA* (as mimicked in our experimental setup), and that these strains will have a less competitive advantage than established MRSA strains.

Further testing, involving a panel of nine different β-lactams of different subclasses, revealed variations in the levels of protection towards the different β-lactams. This is in line with observations in previous studies (Ubukata et al., 1989; Ballhausen et al., 2014). We determined that the level of protection between

the uninduced to the induced condition (without or with *mecA* expression) ranged from fourfold increase for imipenem to 32-fold increase for cefaclor and cephalexin, while the increase for the other β-lactams resided in between. We found that the observed differences in protection did not seem to correlate with the different β-lactam subclasses. Because it is well-know that the various β-lactams have different affinities for the native staphylococcal PBPs (PBP1-4) (Georgopapadakou et al., 1986; Chambers and Sachdeva, 1990; Chambers et al., 1990), we also included agents with variable affinity characteristics in the panel of tested β-lactams.

The two agents where PBP2a provided the highest degree of protection, cefaclor and cephalexin, are both characterized by having a high specific affinity for PBP3, while the other two selective β-lactams tested, cefotaxime and cefoxitin, are selective to PBP2 and PBP4, respectively, and showed only eightfold increase in MIC. This was surprising and contrasts the findings of Antignac and Tomasz (2009), who compared the MIC of a homogenously resistant MRSA strain with an isogenic strain with a deleted *mecA* and found a more pronounced drop in resistance for PBP2 and PBP4 specific β-lactams compared to PBP3 specific β-lactams. The reason for the discrepancy between the studies are unknown. It can be speculated that strain-specific differences could be affecting the results. Certainly, host-specific factors beyond PBP2a are important for the resistance mechanism, but more research is needed to elucidate the mechanisms behind the observed variations.

It is clear that the interplay between the different β-lactam antimicrobials and methicillin resistant staphylococci is complex and even though these mechanisms have been the subject of research for many years there are still pieces of the puzzle to uncover. The variation in the level of protection against the different β-lactams made us speculate whether this could be used as a basis for evolving new schemes for treating β-lactam resistant staphylococci. A limited number of new antimicrobials are reaching the market, which means that there is a need to optimize the use of currently available drugs in addition to developing new substances. If the observed variations in MIC also exist in some clinical isolates, it might be possible, by determining the MIC of the pathogenic strain and considering

the achievable drug concentration at the site of infection, to use specific β-lactams to treat even methicillin resistant isolates as a kind of strain-specific treatment.

4.2.2. Cell-to-cell variation regarding cell fate upon exposure to β lactams

To further examine how heterologous expression of *mecA* protects the *S. aureus* RN4220 cells toward β-lactams on a single-cell level, we performed microfluidics fluorescence time-lapse microscopy. Single-cell growth of a strain expressing *mecA* and a control strain expressing a non-functional *mecA* (point mutation in the active site, rendering the transpeptidase inactive) were studied in culture medium where the cells were exposed to cefoxitin concentrations of 2 or 20 μg/ml, corresponding to concentrations below and above the MIC of the *mecA*-expressing strain. At the lower concentration of cefoxitin, the control strain stopped dividing, while the strain with the functional PBP2a grew normally. At the highest concentration of cefoxitin, both strains stopped dividing. However, the majority of cells did not lyse, showing that there is cell-to-cell variation regarding lysis upon cefoxitin exposure. To test if this was a cefoxitin-specific observation, the same experiment was performed with the 3rd generation cephalosporin cefotaxime, and only a small fraction of the cells lysed during the four hours.

For the cefotaxime experiments, we also changed to cefotaxime-free medium after four hours of antibiotic exposure. There was no regrowth of control cells when they were changed to cefotaxime-free medium for either of the concentrations, indicating that, as expected, all cells were killed by the β-lactam even though most cells did not lyse. Notably, while the *mecA*-positive strain was not able to grow at the highest concentration of cefotaxime (fivefold higher than the MIC of the *mecA*-expressing strain), some of these cells were able to regrow after changing to cefotaxime-free medium after four hours. Thus, again, there may be cell-to-cell variation in the population.

The results suggest that some of the cells can regrow even after exposure to high doses of β-lactams and highlight the importance of continuing to evaluate

duration of exposure and achievable drug concentrations in the treatment of animals and humans to avoid reemergence of infection. The traditional dosing regimen has been a fixed dose over a certain time. While this mostly leads to therapeutic success, the daily dose of the agent often falls in between the MIC of the susceptible and resistant bacteria. Four hours of exposure to the antimicrobial agent is short in a clinical setting, but an initial high dose followed by a lower dose for a period might be an optimal dosing regimen (Paterson et al., 2016).

The initial rise in antimicrobial concentration to above MIC for the resistant population could remove the selective advantage of the traditional treatment (Paterson et al., 2016). It is also possible that even though some of the cells in our experiment were able to regrow, the eradication of most cells could give the immune system a chance to clear the remaining infection, which could enable the use of β-lactam even for MRSA. However, the toxicity and the pharmacokinetics and pharmacodynamics of the agent in question are essential and reaching a sufficiently high concentration might not be feasible.

Our experiment was done in a laboratory-*S. aureus* strain, with unknown relevance to wild-type MRSA, particularly since the levels of resistance in these strains are normally much higher. Nevertheless, the results further substantiate the fact that the interaction between β-lactams and methicillin resistant staphylococci is complex. The results create a foundation for further investigation into the mechanisms of methicillin resistance and the variations observed indicates that there are still unknown factors contributing to this intricate interplay.

4.2.3. Antimicrobial resistance in bovine staphylococci

Mastitis is an important disease in dairy cattle, affecting animal health, welfare and dairy farm economy (Halasa et al., 2007; Rollin et al., 2015) and staphylococci are considered as some of the most central mastitis pathogens (Olde Riekerink et al., 2008; Pyörälä and Taponen, 2009; Peton and Le Loir, 2014; De Visscher et al., 2016). Antimicrobial resistance could complicate the picture further, decreasing treatment success and exacerbating the implications for the animal and the farmer.

The problems associated with antimicrobial resistance are not just related to the animal and farmer, but can potentially have far wider implications. It is estimated that 700 000 people die every year due to resistant infections (O'Neill, 2016), and antimicrobial resistance genes can be transferred between strains and even species by horizontal gene transfer (Schwarz et al., 2014; Partridge et al., 2018). Several staphylococcal species can colonize and infect different host species (Trostle et al., 2001; Otto, 2009; Becker et al., 2014b; Artursson et al., 2016), and it is obvious that monitoring antimicrobial resistance and ensuring up-to-date knowledge is important in all areas where antimicrobials are used. It is important that appropriate measures are implemented to counteract the development and spread of resistance, and that these measures are developed in a One Health perspective.

In paper II we investigated the phenotypic antimicrobial resistance of staphylococci from bovine milk samples from three distinct groups as well as the occurrence of methicillin resistance genes *mecA* and *mecC*. In paper III a selection of these isolates was whole genome sequenced to determine the carriage of antimicrobial resistance genes. Both phenotypic resistance and genotypic resistance were commonly observed in several NAS species.

The results from paper II showed that phenotypic resistance was frequently observed in both *S. epidermidis* and *S. haemolyticus* in all three sample groups (Group 1: Norway, Group 2: Flanders, Belgium and Group 3: Wallonia, Belgium), including multidrug resistant isolates of both species. Multidrug resistant *S. epidermidis* and *S. haemolyticus* of both animal and human origin have been reported previously (Anthonisen et al., 2002; Lee et al., 2018; Nobrega et al., 2018a). In *S. aureus* many of the phenotypic resistance observations were associated with the isolates positive in the PCR for the *mecA* gene. The *mecA* positive *S. aureus* isolates had six to eight observations of resistance, while the remaining *S. aureus* isolates that showed phenotypic resistance had one or two observations of resistance. Regarding the antimicrobial resistance genes detected by WGS, these were also frequently observed in several different NAS species. Both *S. epidermidis* and *S. haemolyticus* isolates contained several resistance genes, but

isolates representing several other species, including *S. chromogenes, S. equorum, S. cohnii* and *S. warneri*, contained more resistance genes in single isolates. The highest number of resistance genes being found in a *S. chromogenes* isolate with seven genes. Overall, in NAS, many antimicrobial resistance genes were found in isolates from one phylogenetic clade containing the species *S. hominis, S. epidermidis, S. haemolyticus, S. hominis* and *S. warneri*. However, the number of isolates in the different species varied which could have affected our results and made it difficult to draw conclusions regarding species differences. For most *S. aureus* isolates we did not detect any antimicrobial resistance genes and in the WGS results the detected resistance genes were also traced to the five isolates containing *mecA*. The findings of multidrug resistant MRSA concurs with previous studies (Vanderhaeghen et al., 2010; Bardiau et al., 2013), and confirms the serious problem of MRSA and multidrug resistance.

Comparing the phenotypic susceptibility testing results with the results from WGS showed that in almost all isolates, and for several frequently occurring resistance genes, such as *blaZ*, *erm*-genes, *mecA* and *tet*-genes, the presence of a resistance gene was associated with phenotypic resistance. Several studies have revealed a high degree of concordance between the phenotypic resistance and the content of resistance genes discovered with WGS in *S. aureus* (Holden et al., 2013; Gordon et al., 2014; Bradley et al., 2015). There are fewer studies on the prediction of phenotypic antimicrobial resistance from WGS for NAS species (Nobrega et al., 2018b). However, both the results of Nobrega et al. (2018b) and our results indicate that it might be possible to use WGS to predict antimicrobial resistance in NAS as well.

The findings of antimicrobial resistant staphylococci in bovine milk and especially multidrug resistant isolates and the fact that staphylococci can colonize and infect different hosts and tissues raise an important question. Antimicrobial resistance has been present in bacteria for millions of years (Aminov, 2009), but the selection pressure that has arisen due to the use of antimicrobials in agriculture and medicine escalated the development (Tacconelli, 2009; Holmes et al., 2016).

The question is whether bovine mastitis should be treated with antimicrobials? For bacteria associated with mastitis the relationship between the use of antimicrobials and the subsequent development of resistance is not entirely clear. Studies of *S. aureus* and NAS from bovine mastitis have indicated a low occurrence of antimicrobial resistance (Bengtsson et al., 2009; Botrel et al., 2010; Persson et al., 2011). In our study, we found antimicrobial resistance in several NAS isolates and in MRSA, but except the MRSA isolates there was a low occurrence of resistance in *S. aureus*. NAS can reside as commensals on the udder skin, hair, nares and vagina, as well as in the environment (Taponen et al., 2008; Piessens et al., 2011; De Visscher et al., 2014), which causes them to be frequently exposed to antimicrobial pressure (Kadlec et al., 2012), possibly driving resistance in NAS.

In paper II resistance to penicillin was most frequently observed in Group 1 (Norway), and was also common in Group 2 (Flanders, Belgium) and 3 (Wallonia, Belgium). The use of some antimicrobials (such as penicillin) is associated with resistance, while the use of others is not (Pol and Ruegg, 2007). The use of systemically administered penicillin and 3rd generation cephalosporins is associated with antimicrobial resistance in NAS, compared to intramammary treatment (Nobrega et al., 2018a). This shows that there is still a need for research on antimicrobial resistance and the use of different antimicrobials and different administration routes regarding treatment of bovine mastitis.

I believe it is naïve to claim that antimicrobial agents should not be used at all in the treatment of bovine mastitis. Considering the many negative consequences of mastitis, especially for the animal welfare, economy and milk quality, as well as the low occurrence of antimicrobial resistance reported in bovine mastitis pathogens, appropriate antimicrobial treatment is an element in the management of some types of udder infections caused by some bacterial pathogens, such as *S. aureus*, along with preventive measures and good herd management (Ruegg, 2017).

Antimicrobial treatment of NAS is more controversial, as the spontaneous elimination rate of infection is relatively high without treatment, However,

treatment can be considered for persistent NAS infections and infections with moderate or severe symptoms (Pyörälä and Taponen, 2009). To ensure that correct and effective treatment is implemented, knowledge regarding antimicrobial resistance is vital. An effective treatment will enhance animal welfare by reducing pain and suffering related to infection, as well as decreasing the economic losses for the farmer and ensuring the food supply. Milk contains several important nutrients, are a central part of many people's diet and many products are easily affordable (Drewnowski, 2011), making dairy production an important industry. To have a well-working dairy industry, it is essential to ensure animal welfare, both for ethical and economic reasons.

Although the occurrence of antimicrobial resistance is low in staphylococci associated with mastitis, it is important to be vigilant. The intertwining microbial ecosystems of animals and humans include, among other, staphylococci, increasing potential unwanted consequences of antimicrobial resistance in staphylococci. Many antimicrobials are used in both human and veterinary medicine and as resistance genes can be transferred between staphylococci and staphylococci can infect several host species, it is obvious that there is a need for a One Health approach to the ongoing antimicrobial resistance pandemic. As we found MRSA in our samples and several of the NAS species showed phenotypic antimicrobial resistance and carried antimicrobial resistance genes, it is natural to wonder if this could be a threat for consumers of dairy products. Antimicrobial resistance in pathogens in milk will likely not be a problem for the consumers of pasteurized milk and products of pasteurized milk. However, there is a trend for consuming raw milk among some people and consuming contaminated, unpasteurized milk comes with the risk of transmission of antimicrobial resistant bacteria and/or foodborne pathogens (Oliver and Murinda, 2012).

4.2.4. Virulence in bovine staphylococci

The virulence of bovine associated *S. aureus* has been well-studied (Monistero et al., 2018; Zhang et al., 2018; Käppeli et al., 2019; Hoekstra et al., 2020), and

although some studies of the virulence of bovine NAS have been published in recent years (Åvall-Jääskeläinen et al., 2018; Naushad et al., 2019; Wuytack et al., 2020), the subject remains unclear. The possibility to use virulence characteristics and the content of virulence genes to guide treatment is an interesting thought, with the prospect of targeted treatment against more virulent or transmissible species or strains.

With regards to mastitis associated *S. aureus* or NAS, *S. aureus* is believed to be more virulent and regularly treated with antimicrobials, while the use of antimicrobials against NAS is more restrained as they often cause subclinical or mild mastitis and the spontaneous elimination rate of NAS infections, without treatment, is relatively high (Pyörälä and Taponen, 2009). Even so, with the notion that NAS is not a uniform group, investigating the virulence potential of NAS, compared to *S. aureus*, is important, as there could be species differences in NAS group with regards to virulence potential.

In paper II we studied the presence of nine virulence genes in three groups of staphylococci collected from bovine milk samples, while in paper III a selection of these isolates was subjected to whole genome sequencing to determine the content of a broad range of different virulence genes. All but one *S. aureus* isolate in paper II carried at least three of the selected virulence genes, while only eight NAS isolates carried any of the virulence genes. This agrees with the view of a lower virulence potential in NAS and their debatable role as udder pathogens. However, the virulence genes analyzed in paper II were selected from virulence genes common in *S. aureus*, and this might lead to a lower detection in NAS. In paper III we investigated the content of virulence genes using WGS, by doing similarity searches for a total of 191 staphylococcal virulence genes, associated with different aspects of bacterial colonization, infection and survival, such as adherence, exoenzymes, host immune evasion, iron uptake and metabolism and several different toxins. We found that the *S. aureus* isolates contained a much larger proportion of virulence genes, compared to NAS, further substantiating the lower virulence potential in NAS.

When comparing the results for *S. aureus* from PCR and from WGS we found that for some genes there was agreement between the results obtained with the two methods, while there were quite large discrepancies for others. For genes such as *tsst* (toxic shock syndrome toxin) and *hla* (alpha hemolysin) the results matched quite well, although with WGS we detected *hla* in all isolates, while the proportion of *hla* positive isolates was somewhat lower by PCR. This may be explained by a higher resolution in WGS and that PCR will only detect the sequence matching the primers. Identification of a larger proportion of genes with WGS compared to PCR has previously been shown for antimicrobial resistance genes (Shelburne et al., 2017). For some of the other genes the results were more surprising: *hlb*, *clfA* and *fnbpA* were detected in the majority of *S. aureus* isolates with PCR (Figure X), but none were detected with WGS. The differences in resolution cannot explain this variation in detection, as PCR only detects known sequences matching the primers, while one would expect WGS to identify a larger number of similar sequences. It is possible that there was an error in the sequence used for similarity search in WGS, leading to the percent identity falling below the limit for inclusion. As the Naushad et al. (2019) database used in paper III was intended for use in NAS isolates, it is also possible that the sequences does not match the genes in *S. aureus* in a degree high enough to get above the limit for inclusion. The fact that VirulenceFinder also did not detect these genes could be due to a stricter threshold for identity. The reason behind this discrepancy in detection warrants further investigation. Anyway, it was surprising to find neither *hlb, clfA* nor *fnbpA* in *S. aureus* in the results from WGS, as these genes have been shown to be prevalent in *S. aureus* from bovine mastitis (Wang et al., 2016).

We also looked at the total virulence gene content in *S. aureus* and the different NAS species and the differences in virulence gene content for NAS species that are considered associated with the environment or being more host-adapted. There was a clear distinction in the total gene content of *S. aureus* compared to NAS, as *S. aureus* had on average more than five times as many virulence genes as most NAS species, emphasizing the great virulence potential of *S. aureus*.

Several studies have indicated that *S. chromogenes*, *S. simulans* and *S. xylosus* have a greater impact on udder health compared to other NAS species (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2019). We did detect a higher content of virulence genes in *S. chromogenes* (on average 44 virulence genes), mostly due to the presence of exotoxins. The *S. hyicus* isolate stood out with a very high content of virulence genes compared to the other NAS species, mostly due to host immune evasion, exotoxin and enterotoxin genes. In the other end of the spectrum we found *S. sciuri* (on average 13 virulence genes) and *S. hominis* (on average 21 virulence genes). The varying number of virulence genes detected in different NAS species further substantiates the idea that NAS is not a uniform group. Naushad et al. (2019) also found the highest virulence potential (defined as the total number of virulence genes) in *S. chromogenes* and *S. hyicus*. However, when they looked at associations between the total number of virulence genes and disease severity, they could not find a significant connection.

There are most likely numerous other factors affecting virulence, such as factors related to the host, the host's environment and competing microbes, making virulence a complex interaction between microbe, host and environment (Balloux et al., 2018; Naushad et al., 2019) and making a prediction of pathogenicity based purely on the content of virulence genes difficult. The limited knowledge on virulence of bovine NAS, combined with the apparent complexity of virulence makes further studies on the relationship between virulence and clinical impact of these species important. As several studies have pointed to *S. chromogenes* as a species with greater impact on udder health (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2019) and both Naushad et al. (2019) and our study found this species to be among those with the highest virulence potential, further focus on the virulence and pathogenesis of *S. chromogenes* seems wise.

The possibility of using virulence gene content to guide antimicrobial treatment appears to be difficult, due to the many factors involved, not only related to the bacterium, but also the host and environment and uncovering this interplay is essential (Balloux et al., 2018; Naushad et al., 2019). Targeting virulence factors could be another potential approach and alternative to antimicrobial therapy, by

inhibiting the pathogenesis of the bacteria. Inactivating virulence factors could enable the host immune system to fight off the infection more easily. Such drugs could also be used in combination with antimicrobial therapy, to ease the effects of bacterial virulence and help the immune system, while antimicrobial drugs work to clear the infection (Cegelski et al., 2008). Either way, continued knowledge regarding antimicrobial resistance and bacterial virulence is vital.

5. Conclusions

The work presented in this thesis have shed light on the mechanisms of β-lactam resistance in *S. aureus* and the interplay between PBP2a and different β-lactam antimicrobials. We showed that the level of resistance conferred by *mecA* in *S. aureus* RN4220 was relatively low compared to wildtype MRSA strains and the resistance towards different β-lactams varied substantially. The highest level of resistance was observed against PBP3-targeting β-lactams, contrasting what have been reported for MRSA strains. In addition, the results of the single-cell microfluidics indicated that some *mecA* expressing cells can survive exposure to concentrations fivefold higher than MIC and regrow when no longer exposed to antimicrobials, highlighting the importance of achievable drug concentrations and duration of exposure in the treatment of infections.

Another achievement is an increased knowledge base on antimicrobial resistance and virulence in bovine staphylococci, especially knowledge regarding NAS. We observed a higher frequency of both phenotypic antimicrobial resistance and antimicrobial resistance genes in bovine NAS compared to *S. aureus*. Among the *S. aureus* isolates, many of the observed antimicrobial resistance characteristics were found in MRSA isolates. Regarding the virulence potential, *S. aureus* stood out with a large content of virulence genes, clearly showing the great virulence potential of this species. Differences between the virulence potential of the NAS species were also seen, with *S. chromogenes* and *S. hyicus* having a higher virulence potential, showing that, in terms of virulence, NAS is not a uniform group.

The findings support the view of a great diversity within the genus *Staphylococcus*, substantiating the differences between *S. aureus* and NAS, while also highlighting differences within NAS and in a population of *mecA*-expressing *S. aureus*. The results could have implications for antimicrobial treatment strategies for staphylococcal infections and demonstrates the need to adapt treatment regimen to the bacterial species in question and its antimicrobial resistance and virulence characteristics.

6. Future perspectives

The emerging, silent pandemic of antimicrobial resistance means that both veterinary and human medicine must ensure a prudent and sustainable use of antimicrobials, implement preventive measures and work to find alternatives to antimicrobial treatment. In order to have effective preventive measures and treatments against bacterial infections, further research on the mechanisms of antimicrobial resistance and virulence is needed. More information about the prevalence and spread of antimicrobial resistance is central. It is not possible to know how implemented measures work without continued monitoring. As the use of WGS continues to increase, studies investigating the concordance between WGS results and clinical resistance in NAS would be useful. Studies on the interactions between different antimicrobials, doses and administration routes and subsequent development of resistance in staphylococci would provide valuable insight into optimal treatment schemes. In light of One Health, studies on horizontal gene transfer and transmission of staphylococci between different host species should be a focus. Studies similar to paper I performed in wildtype MSSA strains will provide further information on how PBP2a mediates β-lactam resistance in *S. aureus*. Additional studies to explore the reasons for the high level of resistance observed for PBP3-targeting β-lactams should be performed.

Investigation into the interaction between microbes and microbial genetics and their interaction with host and host genetics is crucial for unraveling the complex matter of virulence. More studies looking at the content and expression of virulence genes in bovine NAS should be performed, preferably in relation to clinical manifestations, as well as exploring their interplay with the host's immune system. With several indications that *S. chromogenes* could have a greater impact on udder health than many other NAS species and findings of a higher virulence potential in this species, an increased attention on *S. chromogenes* should possibly be prioritized. Studies of differences in susceptibility for NAS infections would further increase the understanding of virulence of bovine NAS and their interactions

with the bovine host and contribute to determining the role of NAS in bovine mastitis and udder health.

7. References

Aarestrup, F.M., Larsen, H.D., Eriksen, N.H., Elsberg, C.S., Jensen, N.E., 1999. Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. Apmis. 107, 425-430.

Abraham, E.P., 1987. Cephalosporins 1945-1986. Drugs. 34 Suppl 2, 1-14. https://doi.org/10.2165/00003495-198700342-00003.

Abraham, E.P., Chain, E., 1988. An enzyme from bacteria able to destroy penicillin. 1940. Rev Infect Dis. 10, 677-678.

Abril, A.G., Villa, T.G., Barros-Velázquez, J., Cañas, B., Sánchez-Pérez, A., Calo-Mata, P., Carrera, M., 2020. *Staphylococcus aureus* Exotoxins and Their Detection in the Dairy Industry and Mastitis. Toxins (Basel). 12https://doi.org/10.3390/toxins12090537.

Aminov, R.I., 2009. The role of antibiotics and antibiotic resistance in nature. Environ Microbiol. 11, 2970-2988. https://doi.org/10.1111/j.1462-2920.2009.01972.x.

Andrews, J.M., 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 48 Suppl 1, 5-16. https://doi.org/10.1093/jac/48.suppl_1.5.

Anthonisen, I.L., Sunde, M., Steinum, T.M., Sidhu, M.S., Sørum, H., 2002. Organization of the antiseptic resistance gene *qacA* and Tn552-related beta-lactamase genes in multidrug- resistant *Staphylococcus haemolyticus* strains of animal and human origins. Antimicrob Agents Chemother. 46, 3606-3612. https://doi.org/10.1128/aac.46.11.3606-3612.2002.

Antignac, A., Tomasz, A., 2009. Reconstruction of the phenotypes of methicillin-resistant *Staphylococcus aureus* by replacement of the staphylococcal cassette chromosome mec with a plasmid-borne copy of *Staphylococcus sciuri pbpD* gene. Antimicrob Agents Chemother. 53, 435-441. https://doi.org/10.1128/aac.01099-08.

Argemi, X., Hansmann, Y., Prola, K., Prévost, G., 2019. Coagulase-Negative Staphylococci Pathogenomics. Int J Mol Sci. 20https://doi.org/10.3390/ijms20051215.

Artursson, K., Söderlund, R., Liu, L., Monecke, S., Schelin, J., 2016. Genotyping of *Staphylococcus aureus* in bovine mastitis and correlation to phenotypic characteristics. Vet Microbiol. 193, 156-161. https://doi.org/10.1016/j.vetmic.2016.08.012.

Baba, T., Kuwahara-Arai, K., Uchiyama, I., Takeuchi, F., Ito, T., Hiramatsu, K., 2009. Complete genome sequence of *Macrococcus caseolyticus* strain JCSCS5402, [corrected] reflecting the ancestral genome of the human-pathogenic staphylococci. J Bacteriol. 191, 1180-1190. https://doi.org/10.1128/jb.01058-08.

Balasubramanian, D., Harper, L., Shopsin, B., Torres, V.J., 2017. *Staphylococcus aureus* pathogenesis in diverse host environments. Pathog Dis. 75https://doi.org/10.1093/femspd/ftx005.

Ballhausen, B., Kriegeskorte, A., Schleimer, N., Peters, G., Becker, K., 2014. The *mecA* homolog *mecC* confers resistance against β-lactams in *Staphylococcus aureus* irrespective of the genetic strain background. Antimicrob Agents Chemother. 58, 3791-3798. https://doi.org/10.1128/aac.02731-13.

Balloux, F., Brønstad Brynildsrud, O., van Dorp, L., Shaw, L.P., Chen, H., Harris, K.A., Wang, H., Eldholm, V., 2018. From Theory to Practice: Translating Whole-Genome Sequencing (WGS) into the Clinic. Trends Microbiol. 26, 1035-1048. https://doi.org/10.1016/j.tim.2018.08.004.

Bannoehr, J., Guardabassi, L., 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol. 23, 253-266, e251-252. https://doi.org/10.1111/j.1365-3164.2012.01046.x.

Bardiau, M., Yamazaki, K., Duprez, J.N., Taminiau, B., Mainil, J.G., Ote, I., 2013. Genotypic and phenotypic characterization of methicillin-resistant

Staphylococcus aureus (MRSA) isolated from milk of bovine mastitis. Lett Appl Microbiol. 57, 181-186. https://doi.org/10.1111/lam.12099.

Baym, M., Lieberman, T.D., Kelsic, E.D., Chait, R., Gross, R., Yelin, I., Kishony, R., 2016. Spatiotemporal microbial evolution on antibiotic landscapes. Science. 353, 1147-1151. https://doi.org/10.1126/science.aag0822.

Bayot, M.L., Bragg, B.N. 2020. Antimicrobial Susceptibility Testing, In: StatPearls. StatPearls Publishing

Copyright © 2020, StatPearls Publishing LLC., Treasure Island (FL).

Becker, K., Ballhausen, B., Köck, R., Kriegeskorte, A., 2014a. Methicillin resistance in *Staphylococcus* isolates: the "mec alphabet" with specific consideration of *mecC*, a mec homolog associated with zoonotic *S. aureus* lineages. Int J Med Microbiol. 304, 794-804. https://doi.org/10.1016/j.ijmm.2014.06.007.

Becker, K., Heilmann, C., Peters, G., 2014b. Coagulase-negative staphylococci. Clin Microbiol Rev. 27, 870-926. https://doi.org/10.1128/cmr.00109-13.

Becker, K., van Alen, S., Idelevich, E.A., Schleimer, N., Seggewiß, J., Mellmann, A., Kaspar, U., Peters, G., 2018. Plasmid-Encoded Transferable *mecB*-Mediated Methicillin Resistance in *Staphylococcus aureus*. Emerg Infect Dis. 24, 242-248. https://doi.org/10.3201/eid2402.171074.

Bell, B.G., Schellevis, F., Stobberingh, E., Goossens, H., Pringle, M., 2014. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect Dis. 14, 13. https://doi.org/10.1186/1471-2334-14-13.

Bengtsson, B., Unnerstad, H.E., Ekman, T., Artursson, K., Nilsson-Ost, M., Waller, K.P., 2009. Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. Vet Microbiol. 136, 142-149. https://doi.org/10.1016/j.vetmic.2008.10.024.

Bjerketorp, J., Jacobsson, K., Frykberg, L., 2004. The von Willebrand factor-binding protein (vWbp) of *Staphylococcus aureus* is a coagulase. FEMS Microbiol Lett. 234, 309-314. https://doi.org/10.1016/j.femsle.2004.03.040.

Blanco-Picazo, P., Roscales, G., Toribio-Avedillo, D., Gómez-Gómez, C., Avila, C., Ballesté, E., Muniesa, M., Rodríguez-Rubio, L., 2020. Antibiotic Resistance Genes in Phage Particles from Antarctic and Mediterranean Seawater Ecosystems. Microorganisms. 8https://doi.org/10.3390/microorganisms8091293.

Bonfiglio, G., Russo, G., Nicoletti, G., 2002. Recent developments in carbapenems. Expert Opin Investig Drugs. 11, 529-544. https://doi.org/10.1517/13543784.11.4.529.

Booth, M.C., Pence, L.M., Mahasreshti, P., Callegan, M.C., Gilmore, M.S., 2001. Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. Infect Immun. 69, 345-352. https://doi.org/10.1128/iai.69.1.345-352.2001.

Bosch, T., van Luit, M., Pluister, G.N., Frentz, D., Haenen, A., Landman, F., Witteveen, S., van Marm-Wattimena, N., van der Heide, H.G., Schouls, L.M., 2016. Changing characteristics of livestock-associated meticillin-resistant *Staphylococcus aureus* isolated from humans - emergence of a subclade transmitted without livestock exposure, the Netherlands, 2003 to 2014. Euro Surveill. 21https://doi.org/10.2807/1560-7917.Es.2016.21.21.30236.

Bosch, T., Verkade, E., van Luit, M., Landman, F., Kluytmans, J., Schouls, L.M., 2015. Transmission and persistence of livestock-associated methicillin-resistant *Staphylococcus aureus* among veterinarians and their household members. Appl Environ Microbiol. 81, 124-129. https://doi.org/10.1128/aem.02803-14.

Botrel, M.A., Haenni, M., Morignat, E., Sulpice, P., Madec, J.Y., Calavas, D., 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Foodborne Pathog Dis. 7, 479-487. https://doi.org/10.1089/fpd.2009.0425.

Bowden, M.G., Chen, W., Singvall, J., Xu, Y., Peacock, S.J., Valtulina, V., Speziale, P., Höök, M., 2005. Identification and preliminary characterization of cell-wall-anchored proteins of

Staphylococcus epidermidis. Microbiology (Reading). 151, 1453-1464. https://doi.org/10.1099/mic.0.27534-0.

Bradley, P., Gordon, N.C., Walker, T.M., Dunn, L., Heys, S., Huang, B., Earle, S., Pankhurst, L.J., Anson, L., de Cesare, M., Piazza, P., Votintseva, A.A., Golubchik, T., Wilson, D.J., Wyllie, D.H., Diel, R., Niemann, S., Feuerriegel, S., Kohl, T.A., Ismail, N., Omar, S.V., Smith, E.G., Buck, D., McVean, G., Walker, A.S., Peto, T.E., Crook, D.W., Iqbal, Z., 2015. Rapid antibiotic-resistance predictions from genome sequence data for *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Nat Commun. 6, 10063. https://doi.org/10.1038/ncomms10063.

Brown-Jaque, M., Calero-Cáceres, W., Espinal, P., Rodríguez-Navarro, J., Miró, E., González-López, J.J., Cornejo, T., Hurtado, J.C., Navarro, F., Muniesa, M., 2018. Antibiotic resistance genes in phage particles isolated from human faeces and induced from clinical bacterial isolates. Int J Antimicrob Agents. 51, 434-442. https://doi.org/10.1016/j.ijantimicag.2017.11.014.

Bush, K., 2013. Proliferation and significance of clinically relevant β-lactamases. Ann N Y Acad Sci. 1277, 84-90. https://doi.org/10.1111/nyas.12023.

Bush, K., Bradford, P.A., 2016. β-Lactams and β-Lactamase Inhibitors: An Overview. Cold Spring Harb Perspect Med. 6https://doi.org/10.1101/cshperspect.a025247.

Cameron, M., Barkema, H.W., De Buck, J., De Vliegher, S., Chaffer, M., Lewis, J., Keefe, G.P., 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. J Dairy Sci. 100, 2137-2147. https://doi.org/10.3168/jds.2016-12020.

Cameron, M., Perry, J., Middleton, J.R., Chaffer, M., Lewis, J., Keefe, G.P., 2018. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. J Dairy Sci. 101, 590-595. https://doi.org/10.3168/jds.2017-13226.

Capurro, A., Artursson, K., Waller, K.P., Bengtsson, B., Ericsson-Unnerstad, H., Aspán, A., 2009. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and tuf gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. Vet Microbiol. 134, 327-333. https://doi.org/10.1016/j.vetmic.2008.08.028.

Cegelski, L., Marshall, G.R., Eldridge, G.R., Hultgren, S.J., 2008. The biology and future prospects of antivirulence therapies. Nat Rev Microbiol. 6, 17-27. https://doi.org/10.1038/nrmicro1818.

Chambers, H.F., 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 10, 781-791. https://doi.org/10.1128/cmr.10.4.781-791.1997.

Chambers, H.F., Deleo, F.R., 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol. 7, 629-641. https://doi.org/10.1038/nrmicro2200.

Chambers, H.F., Sachdeva, M., 1990. Binding of beta-lactam antibiotics to penicillin-binding proteins in methicillin-resistant *Staphylococcus aureus*. J Infect Dis. 161, 1170-1176. https://doi.org/10.1093/infdis/161.6.1170.

Chambers, H.F., Sachdeva, M., Kennedy, S., 1990. Binding affinity for penicillin-binding protein 2a correlates with in vivo activity of beta-lactam antibiotics against methicillin-resistant *Staphylococcus aureus*. J Infect Dis. 162, 705-710. https://doi.org/10.1093/infdis/162.3.705.

Choo, E.J., 2017. Community-Associated Methicillin-Resistant *Staphylococcus aureus* in Nosocomial Infections. Infect Chemother. 49, 158-159. https://doi.org/10.3947/ic.2017.49.2.158.

Colomer-Lluch, M., Imamovic, L., Jofre, J., Muniesa, M., 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. Antimicrob Agents Chemother. 55, 4908-4911. https://doi.org/10.1128/aac.00535-11.

Condas, L.A.Z., De Buck, J., Nobrega, D.B., Carson, D.A., Naushad, S., De Vliegher, S., Zadoks, R.N., Middleton, J.R., Dufour, S., Kastelic, J.P.,

Barkema, H.W., 2017a. Prevalence of non-aureus staphylococci species causing intramammary infections in Canadian dairy herds. J Dairy Sci. 100, 5592-5612. https://doi.org/10.3168/jds.2016-12478.

Condas, L.A.Z., De Buck, J., Nobrega, D.B., Carson, D.A., Roy, J.P., Keefe, G.P., DeVries, T.J., Middleton, J.R., Dufour, S., Barkema, H.W., 2017b. Distribution of non-*aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. J Dairy Sci. 100, 5613-5627. https://doi.org/10.3168/jds.2016-12479.

Corvaglia, A.R., François, P., Hernandez, D., Perron, K., Linder, P., Schrenzel, J., 2010. A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. Proc Natl Acad Sci U S A. 107, 11954-11958. https://doi.org/10.1073/pnas.1000489107.

Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J.A., Hubner, R.J., Sanches, I.S., Tomasz, A., 1996. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. Microb Drug Resist. 2, 377-391. https://doi.org/10.1089/mdr.1996.2.377.

Couto, N., Monchique, C., Belas, A., Marques, C., Gama, L.T., Pomba, C., 2016. Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period. J Antimicrob Chemother. 71, 1479-1487. https://doi.org/10.1093/jac/dkw029.

Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. Int J Med Microbiol. 303, 287-292. https://doi.org/10.1016/j.ijmm.2013.02.009.

Cuny, C., Arnold, P., Hermes, J., Eckmanns, T., Mehraj, J., Schoenfelder, S., Ziebuhr, W., Zhao, Q., Wang, Y., Feßler, A.T., Krause, G., Schwarz, S., Witte, W., 2017. Occurrence of cfr-mediated multiresistance in staphylococci from veal calves and pigs, from humans at the corresponding farms, and from veterinarians and their family members. Vet Microbiol. 200, 88-94. https://doi.org/10.1016/j.vetmic.2016.04.002.

da Costa, L.B., Rajala-Schultz, P.J., Hoet, A., Seo, K.S., Fogt, K., Moon, B.S., 2014. Genetic relatedness and virulence factors of bovine *Staphylococcus aureus* isolated from teat skin and milk. J Dairy Sci. 97, 6907-6916. https://doi.org/10.3168/jds.2014-7972.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 74, 417-433. https://doi.org/10.1128/mmbr.00016-10.

De Buck, J., Ha, V., Naushad, S., Nobrega, D.B., Luby, C., Middleton, J.R., De Vliegher, S., Barkema, H.W., 2021. Non-*aureus* Staphylococci and Bovine Udder Health: Current Understanding and Knowledge Gaps. Frontiers in Veterinary Science. 8https://doi.org/10.3389/fvets.2021.658031.

De Visscher, A., Piepers, S., Haesebrouck, F., De Vliegher, S., 2016. Intramammary infection with coagulase-negative staphylococci at parturition: Species-specific prevalence, risk factors, and effect on udder health. J Dairy Sci. 99, 6457-6469. https://doi.org/10.3168/jds.2015-10458.

De Visscher, A., Piepers, S., Haesebrouck, F., Supré, K., De Vliegher, S., 2017. Coagulase-negative *Staphylococcus* species in bulk milk: Prevalence, distribution, and associated subgroup- and species-specific risk factors. J Dairy Sci. 100, 629-642. https://doi.org/10.3168/jds.2016-11476.

De Visscher, A., Supré, K., Haesebrouck, F., Zadoks, R.N., Piessens, V., Van Coillie, E., Piepers, S., De Vliegher, S., 2014. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet Microbiol. 172, 466-474. https://doi.org/10.1016/j.vetmic.2014.06.011.

De Vliegher, S., Fox, L.K., Piepers, S., McDougall, S., Barkema, H.W., 2012. Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J Dairy Sci. 95, 1025-1040. https://doi.org/10.3168/jds.2010-4074.

Dinges, M.M., Orwin, P.M., Schlievert, P.M., 2000. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev. 13, 16-34, table of contents. https://doi.org/10.1128/cmr.13.1.16-34.2000.

Dos Santos, D.C., Lange, C.C., Avellar-Costa, P., Dos Santos, K.R., Brito, M.A., Giambiagi-deMarval, M., 2016. *Staphylococcus chromogenes*, a Coagulase-Negative *Staphylococcus* Species That Can Clot Plasma. J Clin Microbiol. 54, 1372-1375. https://doi.org/10.1128/jcm.03139-15.

Drawz, S.M., Bonomo, R.A., 2010. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev. 23, 160-201. https://doi.org/10.1128/cmr.00037-09.

Drewnowski, A., 2011. The contribution of milk and milk products to micronutrient density and affordability of the U.S. diet. J Am Coll Nutr. 30, 422s-428s. https://doi.org/10.1080/07315724.2011.10719986.

Dziewanowska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., Bohach, G.A., 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. Infect Immun. 67, 4673-4678. https://doi.org/10.1128/iai.67.9.4673-4678.1999.

ECDC, EFSA, EMA, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. Efsa j. 15, e04872. https://doi.org/10.2903/j.efsa.2017.4872.

Egan, A.J., Cleverley, R.M., Peters, K., Lewis, R.J., Vollmer, W., 2017. Regulation of bacterial cell wall growth. Febs j. 284, 851-867. https://doi.org/10.1111/febs.13959.

Ellington, M.J., Ekelund, O., Aarestrup, F.M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M.T.G., Hopkins, K.L., Iredell, J., Kahlmeter, G., Köser, C.U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J.M., Samuelsen, Ø., Woodford, N., 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect. 23, 2-22. https://doi.org/10.1016/j.cmi.2016.11.012.

Elstrøm, P., Grøntvedt, C.A., Gabrielsen, C., Stegger, M., Angen, Ø., Åmdal, S., Enger, H., Urdahl, A.M., Jore, S., Steinbakk, M., Sunde, M., 2019. Livestock-Associated MRSA CC1 in Norway; Introduction to Pig Farms, Zoonotic Transmission, and Eradication. Front Microbiol. 10, 139. https://doi.org/10.3389/fmicb.2019.00139.

EUCAST 2018. Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing).

Feld, L., Bay, H., Angen, Ø., Larsen, A.R., Madsen, A.M., 2018. Survival of LA-MRSA in Dust from Swine Farms. Ann Work Expo Health. 62, 147-156. https://doi.org/10.1093/annweh/wxx108.

Fernandes, R., Amador, P., Prudêncio, C., 2013. β-Lactams: chemical structure, mode of action and mechanisms of resistance. Reviews in Medical Microbiology. 24, 7-17. https://doi.org/10.1097/MRM.0b013e3283587727.

Ferrari, R.G., Rosario, D.K.A., Cunha-Neto, A., Mano, S.B., Figueiredo, E.E.S., Conte-Junior, C.A., 2019. Worldwide Epidemiology of Salmonella Serovars in Animal-Based Foods: a Meta-analysis. Appl Environ Microbiol. 85https://doi.org/10.1128/aem.00591-19.

Feßler, A.T., Wang, Y., Wu, C., Schwarz, S., 2018. Mobile macrolide resistance genes in staphylococci. Plasmid. 99, 2-10. https://doi.org/10.1016/j.plasmid.2018.05.001.

Fessler, A.T., Wang, Y., Wu, C., Schwarz, S., 2018. Mobile lincosamide resistance genes in staphylococci. Plasmid. 99, 22-31. https://doi.org/10.1016/j.plasmid.2018.06.002.

Fitzgerald, J.R., 2012. Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. Trends Microbiol. 20, 192-198. https://doi.org/10.1016/j.tim.2012.01.006.

Fleming, A., 1929. Classics in infectious diseases: on the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae by Alexander Fleming, Reprinted from the British Journal of Experimental Pathology 10:226-236, 1929. Rev Infect Dis. 2, 129-139.

Foster, T.J., 2017. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiol Rev. 41, 430-449. https://doi.org/10.1093/femsre/fux007.

Foster, T.J., 2019. Can β-Lactam Antibiotics Be Resurrected to Combat MRSA? Trends Microbiol. 27, 26-38. https://doi.org/10.1016/j.tim.2018.06.005.

Foster, T.J., Geoghegan, J.A., Ganesh, V.K., Höök, M., 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 12, 49-62. https://doi.org/10.1038/nrmicro3161.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol. 3, 722-732. https://doi.org/10.1038/nrmicro1235.

Fry, P.R., Middleton, J.R., Dufour, S., Perry, J., Scholl, D., Dohoo, I., 2014. Association of coagulase-negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. J Dairy Sci. 97, 4876-4885. https://doi.org/10.3168/jds.2013-7657.

Geoghegan, J.A., Foster, T.J., 2017. Cell Wall-Anchored Surface Proteins of *Staphylococcus aureus*: Many Proteins, Multiple Functions. Curr Top Microbiol Immunol. 409, 95-120. https://doi.org/10.1007/82_2015_5002.

Georgopapadakou, N.H., Dix, B.A., Mauriz, Y.R., 1986. Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. Antimicrob Agents Chemother. 29, 333-336. https://doi.org/10.1128/aac.29.2.333.

Georgopapadakou, N.H., Liu, F.Y., 1980. Penicillin-binding proteins in bacteria. Antimicrob Agents Chemother. 18, 148-157. https://doi.org/10.1128/aac.18.1.148.

Gordon, N.C., Price, J.R., Cole, K., Everitt, R., Morgan, M., Finney, J., Kearns, A.M., Pichon, B., Young, B., Wilson, D.J., Llewelyn, M.J., Paul, J., Peto, T.E., Crook, D.W., Walker, A.S., Golubchik, T., 2014. Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome

sequencing. J Clin Microbiol. 52, 1182-1191. https://doi.org/10.1128/jcm.03117-13.

Grimminger, F., Rose, F., Sibelius, U., Meinhardt, M., Pötzsch, B., Spriestersbach, R., Bhakdi, S., Suttorp, N., Seeger, W., 1997. Human endothelial cell activation and mediator release in response to the bacterial exotoxins *Escherichia coli* hemolysin and staphylococcal alpha-toxin. J Immunol. 159, 1909-1916.

Grønvold, A.M., L'Abée-Lund, T.M., Strand, E., Sørum, H., Yannarell, A.C., Mackie, R.I., 2010. Fecal microbiota of horses in the clinical setting: potential effects of penicillin and general anesthesia. Vet Microbiol. 145, 366-372. https://doi.org/10.1016/j.vetmic.2010.03.023.

Grønvold, A.M., Mao, Y., L'Abée-Lund, T.M., Sørum, H., Sivertsen, T., Yannarell, A.C., Mackie, R.I., 2011. Fecal microbiota of calves in the clinical setting: effect of penicillin treatment. Vet Microbiol. 153, 354-360. https://doi.org/10.1016/j.vetmic.2011.05.040.

Hair, P.S., Echague, C.G., Sholl, A.M., Watkins, J.A., Geoghegan, J.A., Foster, T.J., Cunnion, K.M., 2010. Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of *Staphylococcus aureus* and decreases complement-mediated phagocytosis. Infect Immun. 78, 1717-1727. https://doi.org/10.1128/iai.01065-09.

Halasa, T., Huijps, K., Østerås, O., Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: a review. Vet Q. 29, 18-31. https://doi.org/10.1080/01652176.2007.9695224.

Hammerum, A.M., Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. Clin Infect Dis. 48, 916-921. https://doi.org/10.1086/597292.

Hansen, J.E., Ronco, T., Stegger, M., Sieber, R.N., Fertner, M.E., Martin, H.L., Farre, M., Toft, N., Larsen, A.R., Pedersen, K., 2019. LA-MRSA CC398 in Dairy Cattle and Veal Calf Farms Indicates Spillover From Pig Production. Front Microbiol. 10, 2733. https://doi.org/10.3389/fmicb.2019.02733.

Harmsen, D., Claus, H., Witte, W., Rothgänger, J., Claus, H., Turnwald, D., Vogel, U., 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol. 41, 5442-5448. https://doi.org/10.1128/jcm.41.12.5442-5448.2003.

Heikkilä, A.M., Liski, E., Pyörälä, S., Taponen, S., 2018. Pathogen-specific production losses in bovine mastitis. J Dairy Sci. 101, 9493-9504. https://doi.org/10.3168/jds.2018-14824.

Heilbronner, S., Holden, M.T., van Tonder, A., Geoghegan, J.A., Foster, T.J., Parkhill, J., Bentley, S.D., 2011. Genome sequence of *Staphylococcus lugdunensis* N920143 allows identification of putative colonization and virulence factors. FEMS Microbiol Lett. 322, 60-67. https://doi.org/10.1111/j.1574-6968.2011.02339.x.

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat Commun. 10, 1124. https://doi.org/10.1038/s41467-019-08853-3.

Hoekstra, J., Zomer, A.L., Rutten, V., Benedictus, L., Stegeman, A., Spaninks, M.P., Bennedsgaard, T.W., Biggs, A., De Vliegher, S., Mateo, D.H., Huber-Schlenstedt, R., Katholm, J., Kovács, P., Krömker, V., Lequeux, G., Moroni, P., Pinho, L., Smulski, S., Supré, K., Swinkels, J.M., Holmes, M.A., Lam, T., Koop, G., 2020. Genomic analysis of European bovine *Staphylococcus aureus* from clinical versus subclinical mastitis. Sci Rep. 10, 18172. https://doi.org/10.1038/s41598-020-75179-2.

Holden, M.T., Hsu, L.Y., Kurt, K., Weinert, L.A., Mather, A.E., Harris, S.R., Strommenger, B., Layer, F., Witte, W., de Lencastre, H., Skov, R., Westh, H., Zemlicková, H., Coombs, G., Kearns, A.M., Hill, R.L., Edgeworth, J., Gould, I., Gant, V., Cooke, J., Edwards, G.F., McAdam, P.R., Templeton, K.E., McCann, A., Zhou, Z., Castillo-Ramírez, S., Feil, E.J., Hudson, L.O., Enright, M.C., Balloux, F., Aanensen, D.M., Spratt, B.G., Fitzgerald, J.R., Parkhill, J., Achtman, M., Bentley, S.D., Nübel, U., 2013. A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. Genome Res. 23, 653-664. https://doi.org/10.1101/gr.147710.112.

Holmes, A.H., Moore, L.S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., Piddock, L.J., 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 387, 176-187. https://doi.org/10.1016/s0140-6736(15)00473-0.

Inoshima, I., Inoshima, N., Wilke, G.A., Powers, M.E., Frank, K.M., Wang, Y., Bubeck Wardenburg, J., 2011. A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nat Med. 17, 1310-1314. https://doi.org/10.1038/nm.2451.

IWG-SCC, 2009. Classification of staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother. 53, 4961-4967. https://doi.org/10.1128/aac.00579-09.

Jasovský, D., Littmann, J., Zorzet, A., Cars, O., 2016. Antimicrobial resistance-a threat to the world's sustainable development. Ups J Med Sci. 121, 159-164. https://doi.org/10.1080/03009734.2016.1195900.

Jenkins, A., Diep, B.A., Mai, T.T., Vo, N.H., Warrener, P., Suzich, J., Stover, C.K., Sellman, B.R., 2015. Differential expression and roles of *Staphylococcus aureus* virulence determinants during colonization and disease. mBio. 6, e02272-02214. https://doi.org/10.1128/mBio.02272-14.

Joensen, K.G., Scheutz, F., Lund, O., Hasman, H., Kaas, R.S., Nielsen, E.M., Aarestrup, F.M., 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of *verotoxigenic Escherichia*

coli. J Clin Microbiol. 52, 1501-1510. https://doi.org/10.1128/jcm.03617-13.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature. 451, 990-993. https://doi.org/10.1038/nature06536.

Josse, J., Velard, F., Gangloff, S.C., 2015. *Staphylococcus aureus* vs. Osteoblast: Relationship and Consequences in Osteomyelitis. Front Cell Infect Microbiol. 5, 85. https://doi.org/10.3389/fcimb.2015.00085.

Kadlec, K., Fessler, A.T., Hauschild, T., Schwarz, S., 2012. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect. 18, 745-755. https://doi.org/10.1111/j.1469-0691.2012.03842.x.

Kahlmeter, G., Brown, D.F., Goldstein, F.W., MacGowan, A.P., Mouton, J.W., Osterlund, A., Rodloff, A., Steinbakk, M., Urbaskova, P., Vatopoulos, A., 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J Antimicrob Chemother. 52, 145-148. https://doi.org/10.1093/jac/dkg312.

Kang, H.Y., Moon, D.C., Mechesso, A.F., Choi, J.H., Kim, S.J., Song, H.J., Kim, M.H., Yoon, S.S., Lim, S.K., 2020. Emergence of *cfr*-Mediated Linezolid Resistance in *Staphylococcus aureus* Isolated from Pig Carcasses. Antibiotics (Basel). 9https://doi.org/10.3390/antibiotics9110769.

Kang, M., Ko, Y.P., Liang, X., Ross, C.L., Liu, Q., Murray, B.E., Höök, M., 2013. Collagen-binding microbial surface components recognizing adhesive matrix molecule (MSCRAMM) of Gram-positive bacteria inhibit complement activation via the classical pathway. J Biol Chem. 288, 20520-20531. https://doi.org/10.1074/jbc.M113.454462.

Kaspar, U., Kriegeskorte, A., Schubert, T., Peters, G., Rudack, C., Pieper, D.H., Wos-Oxley, M., Becker, K., 2016. The culturome of the human nose habitats reveals individual bacterial fingerprint patterns. Environ Microbiol. 18, 2130-2142. https://doi.org/10.1111/1462-2920.12891.

Katayama, Y., Zhang, H.Z., Hong, D., Chambers, H.F., 2003. Jumping the barrier to beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol. 185, 5465-5472. https://doi.org/10.1128/jb.185.18.5465-5472.2003.

Kim, C., Milheiriço, C., Gardete, S., Holmes, M.A., Holden, M.T., de Lencastre, H., Tomasz, A., 2012. Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the β-lactam-resistant phenotype. J Biol Chem. 287, 36854-36863. https://doi.org/10.1074/jbc.M112.395962.

Kirby, W.M., 1944. EXTRACTION OF A HIGHLY POTENT PENICILLIN INACTIVATOR FROM PENICILLIN RESISTANT STAPHYLOCOCCI. Science. 99, 452-453. https://doi.org/10.1126/science.99.2579.452.

Kong, K.F., Schneper, L., Mathee, K., 2010. Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. Apmis. 118, 1-36. https://doi.org/10.1111/j.1600-0463.2009.02563.x.

Kong, K.F., Vuong, C., Otto, M., 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol. 296, 133-139. https://doi.org/10.1016/j.ijmm.2006.01.042.

Kranjec, C., Morales Angeles, D., Torrissen Mårli, M., Fernández, L., García, P., Kjos, M., Diep, D.B., 2021. Staphylococcal Biofilms: Challenges and Novel Therapeutic Perspectives. Antibiotics (Basel). 10https://doi.org/10.3390/antibiotics10020131.

Kriegeskorte, A., Peters, G., 2012. Horizontal gene transfer boosts MRSA spreading. Nat Med. 18, 662-663. https://doi.org/10.1038/nm.2765.

Käppeli, N., Morach, M., Corti, S., Eicher, C., Stephan, R., Johler, S., 2019. *Staphylococcus aureus* related to bovine mastitis in Switzerland: Clonal diversity, virulence gene profiles, and antimicrobial resistance of isolates collected throughout 2017. J Dairy Sci. 102, 3274-3281. https://doi.org/10.3168/jds.2018-15317.

Lammers, A., Nuijten, P.J., Smith, H.E., 1999. The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion to and invasion of bovine

mammary gland cells. FEMS Microbiol Lett. 180, 103-109. https://doi.org/10.1111/j.1574-6968.1999.tb08783.x.

Landers, T.F., Cohen, B., Wittum, T.E., Larson, E.L., 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep. 127, 4-22. https://doi.org/10.1177/003335491212700103.

Larrañaga, O., Brown-Jaque, M., Quirós, P., Gómez-Gómez, C., Blanch, A.R., Rodríguez-Rubio, L., Muniesa, M., 2018. Phage particles harboring antibiotic resistance genes in fresh-cut vegetables and agricultural soil. Environ Int. 115, 133-141. https://doi.org/10.1016/j.envint.2018.03.019.

Lee, J.H., 2003. Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol. 69, 6489-6494. https://doi.org/10.1128/aem.69.11.6489-6494.2003.

Lee, J.Y.H., Monk, I.R., Gonçalves da Silva, A., Seemann, T., Chua, K.Y.L., Kearns, A., Hill, R., Woodford, N., Bartels, M.D., Strommenger, B., Laurent, F., Dodémont, M., Deplano, A., Patel, R., Larsen, A.R., Korman, T.M., Stinear, T.P., Howden, B.P., 2018. Global spread of three multidrug-resistant lineages of *Staphylococcus epidermidis*. Nat Microbiol. 3, 1175-1185. https://doi.org/10.1038/s41564-018-0230-7.

Li, D., Wang, Y., Schwarz, S., Cai, J., Fan, R., Li, J., Feßler, A.T., Zhang, R., Wu, C., Shen, J., 2016. Co-location of the oxazolidinone resistance genes *optrA* and *cfr* on a multiresistance plasmid from *Staphylococcus sciuri*. J Antimicrob Chemother. 71, 1474-1478. https://doi.org/10.1093/jac/dkw040.

Liew, A.T.F., Theis, T., Jensen, S.O., Garcia-Lara, J., Foster, S.J., Firth, N., Lewis, P.J., Harry, E.J., 2011. A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in *Staphylococcus aureus*. Microbiology (Reading). 157, 666-676. https://doi.org/10.1099/mic.0.045146-0.

Linares, J.F., Gustafsson, I., Baquero, F., Martinez, J.L., 2006. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci U S A. 103, 19484-19489. https://doi.org/10.1073/pnas.0608949103.

Lowder, B.V., Guinane, C.M., Ben Zakour, N.L., Weinert, L.A., Conway-Morris, A., Cartwright, R.A., Simpson, A.J., Rambaut, A., Nübel, U., Fitzgerald, J.R., 2009. Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. Proc Natl Acad Sci U S A. 106, 19545-19550. https://doi.org/10.1073/pnas.0909285106.

Lowy, F.D., 1998. *Staphylococcus aureus* infections. N Engl J Med. 339, 520-532. https://doi.org/10.1056/nejm199808203390806.

Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L., Zhang, Q., 2005. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc Natl Acad Sci U S A. 102, 541-546. https://doi.org/10.1073/pnas.0408966102.

Löfblom, J., Kronqvist, N., Uhlén, M., Ståhl, S., Wernérus, H., 2007. Optimization of electroporation-mediated transformation: *Staphylococcus carnosus* as model organism. J Appl Microbiol. 102, 736-747. https://doi.org/10.1111/j.1365-2672.2006.03127.x.

Mahato, S., Mistry, H.U., Chakraborty, S., Sharma, P., Saravanan, R., Bhandari, V., 2017. Identification of Variable Traits among the Methicillin Resistant and Sensitive Coagulase Negative Staphylococci in Milk Samples from Mastitic Cows in India. Front Microbiol. 8, 1446. https://doi.org/10.3389/fmicb.2017.01446.

Malachowa, N., DeLeo, F.R., 2010. Mobile genetic elements of *Staphylococcus aureus*. Cell Mol Life Sci. 67, 3057-3071. https://doi.org/10.1007/s00018-010-0389-4.

Martinez, J.L., 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc Biol Sci. 276, 2521-2530. https://doi.org/10.1098/rspb.2009.0320.

Matuschek, E., Brown, D.F., Kahlmeter, G., 2014. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its

implementation in routine microbiology laboratories. Clin Microbiol Infect. 20, O255-266. https://doi.org/10.1111/1469-0691.12373.

McAdow, M., Missiakas, D.M., Schneewind, O., 2012. *Staphylococcus aureus* secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. J Innate Immun. 4, 141-148. https://doi.org/10.1159/000333447.

McCarthy, A.J., Lindsay, J.A., 2010. Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. BMC Microbiol. 10, 173. https://doi.org/10.1186/1471-2180-10-173.

Mediavilla, J.R., Chen, L., Mathema, B., Kreiswirth, B.N., 2012. Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Curr Opin Microbiol. 15, 588-595. https://doi.org/10.1016/j.mib.2012.08.003.

Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekalanos, J.J., Kahne, D., Walker, S., Kruse, A.C., Bernhardt, T.G., Rudner, D.Z., 2016. SEDS proteins are a widespread family of bacterial cell wall polymerases. Nature. 537, 634-638. https://doi.org/10.1038/nature19331.

Merialdi, G., Feltrin, F., Gaetarelli, B., Lombardi, G., Iurescia, M., Alba, P., Luppi, A., Martinelli, N., Ramini, M., Carfora, V., Franco, A., Battisti, A., 2019. Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) spa type t127, Sequence Type (ST)1, quickly spreads and persists among young pigs. Pathog Dis. 77https://doi.org/10.1093/femspd/ftz033.

Mir-Sanchis, I., Roman, C.A., Misiura, A., Pigli, Y.Z., Boyle-Vavra, S., Rice, P.A., 2016. Staphylococcal SCCmec elements encode an active MCM-like helicase and thus may be replicative. Nat Struct Mol Biol. 23, 891-898. https://doi.org/10.1038/nsmb.3286.

Monistero, V., Graber, H.U., Pollera, C., Cremonesi, P., Castiglioni, B., Bottini, E., Ceballos-Marquez, A., Lasso-Rojas, L., Kroemker, V., Wente, N., Petzer, I.M., Santisteban, C., Runyan, J., Veiga Dos Santos, M., Alves, B.G., Piccinini, R., Bronzo, V., Abbassi, M.S., Said, M.B., Moroni, P., 2018.

Staphylococcus aureus Isolates from Bovine Mastitis in Eight Countries: Genotypes, Detection of Genes Encoding Different Toxins and Other Virulence Genes. Toxins (Basel). 10https://doi.org/10.3390/toxins10060247.

Monk, I.R., Tree, J.J., Howden, B.P., Stinear, T.P., Foster, T.J., 2015. Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. mBio. 6, e00308-00315. https://doi.org/10.1128/mBio.00308-15.

Mroczkowska, A., Żmudzki, J., Marszałek, N., Orczykowska-Kotyna, M., Komorowska, I., Nowak, A., Grzesiak, A., Czyżewska-Dors, E., Dors, A., Pejsak, Z., Hryniewicz, W., Wyszomirski, T., Empel, J., 2017. Livestock-associated *Staphylococcus aureus* on Polish pig farms. PLoS One. 12, e0170745. https://doi.org/10.1371/journal.pone.0170745.

Munita, J.M., Arias, C.A., 2016. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 4https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.

Murchan, S., Kaufmann, M.E., Deplano, A., de Ryck, R., Struelens, M., Zinn, C.E., Fussing, V., Salmenlinna, S., Vuopio-Varkila, J., El Solh, N., Cuny, C., Witte, W., Tassios, P.T., Legakis, N., van Leeuwen, W., van Belkum, A., Vindel, A., Laconcha, I., Garaizar, J., Haeggman, S., Olsson-Liljequist, B., Ransjo, U., Coombes, G., Cookson, B., 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J Clin Microbiol. 41, 1574-1585. https://doi.org/10.1128/jcm.41.4.1574-1585.2003.

Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., Francois, P., Cheung, A.L., 2011. Whole-genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. J Bacteriol. 193, 2332-2335. https://doi.org/10.1128/jb.00027-11.

Naushad, S., Naqvi, S.A., Nobrega, D., Luby, C., Kastelic, J.P., Barkema, H.W., De Buck, J., 2019. Comprehensive Virulence Gene Profiling of Bovine Non-*aureus* Staphylococci Based on Whole-Genome Sequencing Data. mSystems. 4https://doi.org/10.1128/mSystems.00098-18.

Newton, G.G., Abraham, E.P., 1956. Isolation of cephalosporin C, a penicillin-like antibiotic containing D-alpha-aminoadipic acid. Biochem J. 62, 651-658. https://doi.org/10.1042/bj0620651.

Nguyen, L.T., Vogel, H.J., 2016. Staphylokinase has distinct modes of interaction with antimicrobial peptides, modulating its plasminogen-activation properties. Sci Rep. 6, 31817. https://doi.org/10.1038/srep31817.

Nobrega, D.B., De Buck, J., Barkema, H.W., 2018a. Antimicrobial resistance in non-aureus staphylococci isolated from milk is associated with systemic but not intramammary administration of antimicrobials in dairy cattle. J Dairy Sci. 101, 7425-7436. https://doi.org/10.3168/jds.2018-14540.

Nobrega, D.B., Naushad, S., Naqvi, S.A., Condas, L.A.Z., Saini, V., Kastelic, J.P., Luby, C., De Buck, J., Barkema, H.W., 2018b. Prevalence and Genetic Basis of Antimicrobial Resistance in Non-*aureus* Staphylococci Isolated from Canadian Dairy Herds. Front Microbiol. 9, 256. https://doi.org/10.3389/fmicb.2018.00256.

NORM/NORM-VET, 2019. 'Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway'. Tromsø/Oslo 2020.

Norwegian Medicines Agency, 2012. 'Terapianbefaling: Bruk av antibakterielle midler til produksjonsdyr'.

Nygaard, T.K., Pallister, K.B., DuMont, A.L., DeWald, M., Watkins, R.L., Pallister, E.Q., Malone, C., Griffith, S., Horswill, A.R., Torres, V.J., Voyich, J.M., 2012. Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA300 infection. PLoS One. 7, e36532. https://doi.org/10.1371/journal.pone.0036532.

O'Neill, J., 2016. 'Tackling Drug-Resistant Infections Globally: final report and recommendations'. May 19 2016.

Ogston, A., 1882. Micrococcus Poisoning. J Anat Physiol. 17, 24-58.

OHC 2021. What is One Health? (One Health Commision).

Olde Riekerink, R.G., Barkema, H.W., Kelton, D.F., Scholl, D.T., 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J Dairy Sci. 91, 1366-1377. https://doi.org/10.3168/jds.2007-0757.

Oliver, S.P., Murinda, S.E., 2012. Antimicrobial resistance of mastitis pathogens. Vet Clin North Am Food Anim Pract. 28, 165-185. https://doi.org/10.1016/j.cvfa.2012.03.005.

Osterås, O., Sølverød, L., Reksen, O., 2006. Milk culture results in a large Norwegian survey--effects of season, parity, days in milk, resistance, and clustering. J Dairy Sci. 89, 1010-1023. https://doi.org/10.3168/jds.S0022-0302(06)72167-1.

Ote, I., Taminiau, B., Duprez, J.N., Dizier, I., Mainil, J.G., 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet Microbiol. 153, 285-292. https://doi.org/10.1016/j.vetmic.2011.05.042.

Otero, L.H., Rojas-Altuve, A., Llarrull, L.I., Carrasco-López, C., Kumarasiri, M., Lastochkin, E., Fishovitz, J., Dawley, M., Hesek, D., Lee, M., Johnson, J.W., Fisher, J.F., Chang, M., Mobashery, S., Hermoso, J.A., 2013. How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. Proc Natl Acad Sci U S A. 110, 16808-16813. https://doi.org/10.1073/pnas.1300118110.

Otto, M., 2009. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat Rev Microbiol. 7, 555-567. https://doi.org/10.1038/nrmicro2182.

Partridge, S.R., Kwong, S.M., Firth, N., Jensen, S.O., 2018. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 31https://doi.org/10.1128/cmr.00088-17.

Paterson, G.K., Harrison, E.M., Holmes, M.A., 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 22, 42-47. https://doi.org/10.1016/j.tim.2013.11.003.

Paterson, I.K., Hoyle, A., Ochoa, G., Baker-Austin, C., Taylor, N.G., 2016. Optimising Antibiotic Usage to Treat Bacterial Infections. Sci Rep. 6, 37853. https://doi.org/10.1038/srep37853.

Patz, J.A., Daszak, P., Tabor, G.M., Aguirre, A.A., Pearl, M., Epstein, J., Wolfe, N.D., Kilpatrick, A.M., Foufopoulos, J., Molyneux, D., Bradley, D.J., 2004. Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence. Environ Health Perspect. 112, 1092-1098. https://doi.org/10.1289/ehp.6877.

Peacock, S.J., Paterson, G.K., 2015. Mechanisms of Methicillin Resistance in *Staphylococcus aureus*. Annu Rev Biochem. 84, 577-601. https://doi.org/10.1146/annurev-biochem-060614-034516.

Peetermans, M., Verhamme, P., Vanassche, T., 2015. Coagulase Activity by *Staphylococcus aureus*: A Potential Target for Therapy? Semin Thromb Hemost. 41, 433-444. https://doi.org/10.1055/s-0035-1549849.

Persing, D.H., 1991. Polymerase chain reaction: trenches to benches. J Clin Microbiol. 29, 1281-1285. https://doi.org/10.1128/jcm.29.7.1281-1285.1991.

Persson, Y., Nyman, A.K., Grönlund-Andersson, U., 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Vet Scand. 53, 36. https://doi.org/10.1186/1751-0147-53-36.

Peton, V., Le Loir, Y., 2014. *Staphylococcus aureus* in veterinary medicine. Infect Genet Evol. 21, 602-615. https://doi.org/10.1016/j.meegid.2013.08.011.

Pickering, A.C., Fitzgerald, J.R., 2020. The Role of Gram-Positive Surface Proteins in Bacterial Niche- and Host-Specialization. Front Microbiol. 11, 594737. https://doi.org/10.3389/fmicb.2020.594737.

Piessens, V., Van Coillie, E., Verbist, B., Supré, K., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M., De Vliegher, S., 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. J Dairy Sci. 94, 2933-2944. https://doi.org/10.3168/jds.2010-3956.

Piette, A., Verschraegen, G., 2009. Role of coagulase-negative staphylococci in human disease. Vet Microbiol. 134, 45-54. https://doi.org/10.1016/j.vetmic.2008.09.009.

Pol, M., Ruegg, P.L., 2007. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. J Dairy Sci. 90, 262-273. https://doi.org/10.3168/jds.S0022-0302(07)72627-9.

Pottegård, A., Broe, A., Aabenhus, R., Bjerrum, L., Hallas, J., Damkier, P., 2015. Use of antibiotics in children: a Danish nationwide drug utilization study. Pediatr Infect Dis J. 34, e16-22. https://doi.org/10.1097/inf.0000000000000519.

PubMLST 2020. Organisms (Public databases for molecular typing and microbial genome diversity).

Pyörälä, S., Taponen, S., 2009. Coagulase-negative staphylococci-emerging mastitis pathogens. Vet Microbiol. 134, 3-8. https://doi.org/10.1016/j.vetmic.2008.09.015.

Ramirez, M.S., Tolmasky, M.E., 2010. Aminoglycoside modifying enzymes. Drug Resist Updat. 13, 151-171. https://doi.org/10.1016/j.drup.2010.08.003.

Rammelkamp, C.H., Keefer, C.S., 1943. PENICILLIN: ITS ANTIBACTERIAL EFFECT IN WHOLE BLOOD AND SERUM FOR THE HEMOLYTIC *STREPTOCOCCUS* AND *STAPHYLOCOCCUS AUREUS*. J Clin Invest. 22, 649-657. https://doi.org/10.1172/jci101437.

Rodriguez, M., Hogan, P.G., Satola, S.W., Crispell, E., Wylie, T., Gao, H., Sodergren, E., Weinstock, G.M., Burnham, C.A., Fritz, S.A., 2015. Discriminatory Indices of Typing Methods for Epidemiologic Analysis of Contemporary *Staphylococcus aureus* Strains. Medicine (Baltimore). 94, e1534. https://doi.org/10.1097/md.0000000000001534.

Rollin, E., Dhuyvetter, K.C., Overton, M.W., 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Prev Vet Med. 122, 257-264. https://doi.org/10.1016/j.prevetmed.2015.11.006.

Rolo, J., Worning, P., Nielsen, J.B., Bowden, R., Bouchami, O., Damborg, P., Guardabassi, L., Perreten, V., Tomasz, A., Westh, H., de Lencastre, H.,

Miragaia, M., 2017. Evolutionary Origin of the Staphylococcal Cassette Chromosome mec (SCCmec). Antimicrob Agents Chemother. 61https://doi.org/10.1128/aac.02302-16.

Rossen, J.W.A., Friedrich, A.W., Moran-Gilad, J., 2018. Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. Clin Microbiol Infect. 24, 355-360. https://doi.org/10.1016/j.cmi.2017.11.001.

Rouch, D.A., Byrne, M.E., Kong, Y.C., Skurray, R.A., 1987. The aacA-aphD gentamicin and kanamycin resistance determinant of Tn4001 from *Staphylococcus aureus*: expression and nucleotide sequence analysis. J Gen Microbiol. 133, 3039-3052. https://doi.org/10.1099/00221287-133-11-3039.

Ruegg, P.L., 2017. A 100-Year Review: Mastitis detection, management, and prevention. J Dairy Sci. 100, 10381-10397. https://doi.org/10.3168/jds.2017-13023.

Salasia, S.I., Khusnan, Z., Lammler, C., Zschock, M., 2004. Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. J Vet Sci. 5, 103-109.

Sampimon, O.C., Zadoks, R.N., De Vliegher, S., Supré, K., Haesebrouck, F., Barkema, H.W., Sol, J., Lam, T.J., 2009. Performance of API Staph ID 32 and Staph-Zym for identification of coagulase-negative staphylococci isolated from bovine milk samples. Vet Microbiol. 136, 300-305. https://doi.org/10.1016/j.vetmic.2008.11.004.

Schilcher, K., Horswill, A.R., 2020. Staphylococcal Biofilm Development: Structure, Regulation, and Treatment Strategies. Microbiol Mol Biol Rev. 84https://doi.org/10.1128/mmbr.00026-19.

Schnellmann, C., Gerber, V., Rossano, A., Jaquier, V., Panchaud, Y., Doherr, M.G., Thomann, A., Straub, R., Perreten, V., 2006. Presence of new *mecA* and *mph(C)* variants conferring antibiotic resistance in *Staphylococcus* spp.

isolated from the skin of horses before and after clinic admission. J Clin Microbiol. 44, 4444-4454. https://doi.org/10.1128/jcm.00868-06.

Schoenfelder, S.M., Dong, Y., Feßler, A.T., Schwarz, S., Schoen, C., Köck, R., Ziebuhr, W., 2017. Antibiotic resistance profiles of coagulase-negative staphylococci in livestock environments. Vet Microbiol. 200, 79-87. https://doi.org/10.1016/j.vetmic.2016.04.019.

Schwarz, S., Feßler, A.T., Loncaric, I., Wu, C., Kadlec, K., Wang, Y., Shen, J., 2018. Antimicrobial Resistance among Staphylococci of Animal Origin. Microbiol Spectr. 6https://doi.org/10.1128/microbiolspec.ARBA-0010-2017.

Schwarz, S., Kehrenberg, C., Doublet, B., Cloeckaert, A., 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev. 28, 519-542. https://doi.org/10.1016/j.femsre.2004.04.001.

Schwarz, S., Shen, J., Wendlandt, S., Feßler, A.T., Wang, Y., Kadlec, K., Wu, C.M., 2014. Plasmid-Mediated Antimicrobial Resistance in Staphylococci and Other Firmicutes. Microbiol Spectr. 2https://doi.org/10.1128/microbiolspec.PLAS-0020-2014.

Schwendener, S., Cotting, K., Perreten, V., 2017. Novel methicillin resistance gene *mecD* in clinical Macrococcus caseolyticus strains from bovine and canine sources. Sci Rep. 7, 43797. https://doi.org/10.1038/srep43797.

Seguin, J.C., Walker, R.D., Caron, J.P., Kloos, W.E., George, C.G., Hollis, R.J., Jones, R.N., Pfaller, M.A., 1999. Methicillin-resistant *Staphylococcus aureus* outbreak in a veterinary teaching hospital: potential human-to-animal transmission. J Clin Microbiol. 37, 1459-1463. https://doi.org/10.1128/jcm.37.5.1459-1463.1999.

Sendi, P., Proctor, R.A., 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol. 17, 54-58. https://doi.org/10.1016/j.tim.2008.11.004.

Sharkey, L.K., Edwards, T.A., O'Neill, A.J., 2016. ABC-F Proteins Mediate Antibiotic Resistance through Ribosomal Protection. mBio. 7, e01975. https://doi.org/10.1128/mBio.01975-15.

Sharma, C., Rokana, N., Chandra, M., Singh, B.P., Gulhane, R.D., Gill, J.P.S., Ray, P., Puniya, A.K., Panwar, H., 2017. Antimicrobial Resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. Front Vet Sci. 4, 237. https://doi.org/10.3389/fvets.2017.00237.

Shelburne, S.A., Kim, J., Munita, J.M., Sahasrabhojane, P., Shields, R.K., Press, E.G., Li, X., Arias, C.A., Cantarel, B., Jiang, Y., Kim, M.S., Aitken, S.L., Greenberg, D.E., 2017. Whole-Genome Sequencing Accurately Identifies Resistance to Extended-Spectrum β-Lactams for Major Gram-Negative Bacterial Pathogens. Clin Infect Dis. 65, 738-745. https://doi.org/10.1093/cid/cix417.

Sheppard, S.K., Guttman, D.S., Fitzgerald, J.R., 2018. Population genomics of bacterial host adaptation. Nat Rev Genet. 19, 549-565. https://doi.org/10.1038/s41576-018-0032-z.

Shopsin, B., Gomez, M., Montgomery, S.O., Smith, D.H., Waddington, M., Dodge, D.E., Bost, D.A., Riehman, M., Naidich, S., Kreiswirth, B.N., 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol. 37, 3556-3563. https://doi.org/10.1128/jcm.37.11.3556-3563.1999.

Sieber, R.N., Skov, R.L., Nielsen, J., Schulz, J., Price, L.B., Aarestrup, F.M., Larsen, A.R., Stegger, M., Larsen, J., 2018. Drivers and Dynamics of Methicillin-Resistant Livestock-Associated *Staphylococcus aureus* CC398 in Pigs and Humans in Denmark. mBio. 9https://doi.org/10.1128/mBio.02142-18.

Siguier, P., Gagnevin, L., Chandler, M., 2009. The new IS1595 family, its relation to IS1 and the frontier between insertion sequences and transposons. Res Microbiol. 160, 232-241. https://doi.org/10.1016/j.resmic.2009.02.003.

Sinha, B., François, P.P., Nüsse, O., Foti, M., Hartford, O.M., Vaudaux, P., Foster, T.J., Lew, D.P., Herrmann, M., Krause, K.H., 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol. 1, 101-117. https://doi.org/10.1046/j.1462-5822.1999.00011.x.

Smyth, D.S., Wong, A., Robinson, D.A., 2011. Cross-species spread of SCCmec IV subtypes in staphylococci. Infect Genet Evol. 11, 446-453. https://doi.org/10.1016/j.meegid.2010.12.005.

Sollid, J.U., Furberg, A.S., Hanssen, A.M., Johannessen, M., 2014. *Staphylococcus aureus*: determinants of human carriage. Infect Genet Evol. 21, 531-541. https://doi.org/10.1016/j.meegid.2013.03.020.

Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: building the web of life. Nat Rev Genet. 16, 472-482. https://doi.org/10.1038/nrg3962.

Soumya, K.R., Philip, S., Sugathan, S., Mathew, J., Radhakrishnan, E.K., 2017. Virulence factors associated with Coagulase Negative Staphylococci isolated from human infections. 3 Biotech. 7, 140. https://doi.org/10.1007/s13205-017-0753-2.

Spaulding, A.R., Salgado-Pabón, W., Kohler, P.L., Horswill, A.R., Leung, D.Y., Schlievert, P.M., 2013. Staphylococcal and streptococcal superantigen exotoxins. Clin Microbiol Rev. 26, 422-447. https://doi.org/10.1128/cmr.00104-12.

Starlander, G., Börjesson, S., Grönlund-Andersson, U., Tellgren-Roth, C., Melhus, A., 2014. Cluster of infections caused by methicillin-resistant *Staphylococcus pseudintermedius* in humans in a tertiary hospital. J Clin Microbiol. 52, 3118-3120. https://doi.org/10.1128/jcm.00703-14.

Stegger, M., Andersen, P.S., Kearns, A., Pichon, B., Holmes, M.A., Edwards, G., Laurent, F., Teale, C., Skov, R., Larsen, A.R., 2012. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mecA*(LGA251). Clin Microbiol Infect. 18, 395-400. https://doi.org/10.1111/j.1469-0691.2011.03715.x.

Stegmann, R., Burnens, A., Maranta, C.A., Perreten, V., 2010. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius* ST71. J Antimicrob Chemother. 65, 2047-2048. https://doi.org/10.1093/jac/dkq241.

Strommenger, B., Braulke, C., Heuck, D., Schmidt, C., Pasemann, B., Nübel, U., Witte, W., 2008. spa Typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J Clin Microbiol. 46, 574-581. https://doi.org/10.1128/jcm.01599-07.

Supré, K., De Vliegher, S., Sampimon, O.C., Zadoks, R.N., Vaneechoutte, M., Baele, M., De Graef, E., Piepers, S., Haesebrouck, F., 2009. Technical note: use of transfer RNA-intergenic spacer PCR combined with capillary electrophoresis to identify coagulase-negative *Staphylococcus* species originating from bovine milk and teat apices. J Dairy Sci. 92, 3204-3210. https://doi.org/10.3168/jds.2008-1923.

Supré, K., Haesebrouck, F., Zadoks, R.N., Vaneechoutte, M., Piepers, S., De Vliegher, S., 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. J Dairy Sci. 94, 2329-2340. https://doi.org/10.3168/jds.2010-3741.

Sørensen, A.I.V., Jensen, V.F., Boklund, A., Halasa, T., Christensen, H., Toft, N., 2018. Risk factors for the occurrence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in Danish pig herds. Prev Vet Med. 159, 22-29. https://doi.org/10.1016/j.prevetmed.2018.08.012.

Tacconelli, E., 2009. Antimicrobial use: risk driver of multidrug resistant microorganisms in healthcare settings. Curr Opin Infect Dis. 22, 352-358. https://doi.org/10.1097/QCO.0b013e32832d52e0.

Tam, K., Torres, V.J., 2019. *Staphylococcus aureus* Secreted Toxins and Extracellular Enzymes. Microbiol Spectr. 7https://doi.org/10.1128/microbiolspec.GPP3-0039-2018.

Taponen, S., Björkroth, J., Pyörälä, S., 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd. J Dairy Res. 75, 422-429. https://doi.org/10.1017/s0022029908003312.

Taylor, L.H., Latham, S.M., Woolhouse, M.E., 2001. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci. 356, 983-989. https://doi.org/10.1098/rstb.2001.0888.

Thammavongsa, V., Kim, H.K., Missiakas, D., Schneewind, O., 2015. Staphylococcal manipulation of host immune responses. Nat Rev Microbiol. 13, 529-543. https://doi.org/10.1038/nrmicro3521.

Thwaites, G.E., Gant, V., 2011. Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? Nat Rev Microbiol. 9, 215-222. https://doi.org/10.1038/nrmicro2508.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., Fowler, V.G., Jr., 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 28, 603-661. https://doi.org/10.1128/cmr.00134-14.

Trostle, S.S., Peavey, C.L., King, D.S., Hartmann, F.A., 2001. Treatment of methicillin-resistant *Staphylococcus epidermidis* infection following repair of an ulnar fracture and humeroradial joint luxation in a horse. J Am Vet Med Assoc. 218, 554-559, 527. https://doi.org/10.2460/javma.2001.218.554.

Tsubakishita, S., Kuwahara-Arai, K., Baba, T., Hiramatsu, K., 2010a. Staphylococcal cassette chromosome mec-like element in *Macrococcus caseolyticus*. Antimicrob Agents Chemother. 54, 1469-1475. https://doi.org/10.1128/aac.00575-09.

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., Hiramatsu, K., 2010b. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother. 54, 4352-4359. https://doi.org/10.1128/aac.00356-10.

Typas, A., Banzhaf, M., Gross, C.A., Vollmer, W., 2011. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol. 10, 123-136. https://doi.org/10.1038/nrmicro2677.

Ubukata, K., Nonoguchi, R., Matsuhashi, M., Konno, M., 1989. Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant S. aureus-specific penicillin-binding protein. J Bacteriol. 171, 2882-2885. https://doi.org/10.1128/jb.171.5.2882-2885.1989.

Valckenier, D., Piepers, S., De Visscher, A., Bruckmaier, R.M., De Vliegher, S., 2019. Effect of intramammary infection with non-aureus staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. J Dairy Sci. 102, 6442-6453. https://doi.org/10.3168/jds.2018-15913.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., Butaye, P., 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet Microbiol. 144, 166-171. https://doi.org/10.1016/j.vetmic.2009.12.044.

Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F., De Vliegher, S., 2014. Invited review: effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. J Dairy Sci. 97, 5275-5293. https://doi.org/10.3168/jds.2013-7775.

(VKM), Norwegian Scientific Committee for Food and Environment, VKM, Yngvild Wasteson, Blix, H.S., Joner, E., Madslien, E.-H., Ottoson, J., Sørum, H., Uhl, W., Yazdankhah, S., Bergh, Ø., Eklo, O.M., Nielsen, K.M., Trosvik, P., 2020. 'Assessment of the impact of wastewater and sewage sludge treatment methods on antimicrobial resistance'. Oslo, Norway

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W., editors, 2009. Bergey's Manual of Systemic Bacteriology, Volume 3: The Firmicutes. Springer, New York.

Walker, J.N., Flores-Mireles, A.L., Pinkner, C.L., Schreiber, H.L.t., Joens, M.S., Park, A.M., Potretzke, A.M., Bauman, T.M., Pinkner, J.S., Fitzpatrick, J.A.J., Desai, A., Caparon, M.G., Hultgren, S.J., 2017. Catheterization alters bladder ecology to potentiate *Staphylococcus aureus* infection of the urinary tract. Proc Natl Acad Sci U S A. 114, E8721-e8730. https://doi.org/10.1073/pnas.1707572114.

Wang, B., Muir, T.W., 2016. Regulation of Virulence in *Staphylococcus aureus*: Molecular Mechanisms and Remaining Puzzles. Cell Chem Biol. 23, 214-224. https://doi.org/10.1016/j.chembiol.2016.01.004.

Wang, D., Zhang, L., Zhou, X., He, Y., Yong, C., Shen, M., Szenci, O., Han, B., 2016. Antimicrobial susceptibility, virulence genes, and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia, China. J Dairy Sci. 99, 9560-9569. https://doi.org/10.3168/jds.2016-11625.

Waters, C.M., Bassler, B.L., 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 21, 319-346. https://doi.org/10.1146/annurev.cellbio.21.012704.131001.

Weiss, R.A., McMichael, A.J., 2004. Social and environmental risk factors in the emergence of infectious diseases. Nat Med. 10, S70-76. https://doi.org/10.1038/nm1150.

Wendlandt, S., Feßler, A.T., Monecke, S., Ehricht, R., Schwarz, S., Kadlec, K., 2013. The diversity of antimicrobial resistance genes among staphylococci of animal origin. Int J Med Microbiol. 303, 338-349. https://doi.org/10.1016/j.ijmm.2013.02.006.

Wendlandt, S., Shen, J., Kadlec, K., Wang, Y., Li, B., Zhang, W.J., Feßler, A.T., Wu, C., Schwarz, S., 2015. Multidrug resistance genes in staphylococci from animals that confer resistance to critically and highly important antimicrobial agents in human medicine. Trends Microbiol. 23, 44-54. https://doi.org/10.1016/j.tim.2014.10.002.

WHO, 2012. 'The evolving threat of antimicrobial resistance: options for actions.'. Geneva, Switzerland

WHO 2017a. One Health (World Health Organization).

WHO 2017b. WHO publishes list of bacteria for which new antibiotics are urgently needed (Geneva, Switzerland, World Health Organization).

Organization, World Health, WHO, 2019. 'Critically important antimicrobials for human medicine'. Geneva

WHO 2020a. How WHO is working to track down the animal reservoir of the SARS-CoV-2 virus (World Health Organization).

WHO 2020b. Zoonoses (World Health Organization).

Williamson, D.A., Heffernan, H., Nimmo, G., 2015. Contemporary genomic approaches in the diagnosis and typing of *Staphylococcus aureus*. Pathology. 47, 270-275. https://doi.org/10.1097/pat.0000000000000236.

Wilson, G.J., Tuffs, S.W., Wee, B.A., Seo, K.S., Park, N., Connelley, T., Guinane, C.M., Morrison, W.I., Fitzgerald, J.R., 2018. Bovine *Staphylococcus aureus* Superantigens Stimulate the Entire T Cell Repertoire of Cattle. Infect Immun. 86https://doi.org/10.1128/iai.00505-18.

Wiseman, G.M., 1975. The hemolysins of *Staphylococcus aureus*. Bacteriol Rev. 39, 317-344.

Wu, S., Piscitelli, C., de Lencastre, H., Tomasz, A., 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb Drug Resist. 2, 435-441. https://doi.org/10.1089/mdr.1996.2.435.

Wuytack, A., De Visscher, A., Piepers, S., Boyen, F., Haesebrouck, F., De Vliegher, S., 2020. Distribution of non-*aureus* staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential. J Dairy Sci. 103, 10658-10675. https://doi.org/10.3168/jds.2020-18265.

Zadoks, R.N., Watts, J.L., 2009. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. Vet Microbiol. 134, 20-28. https://doi.org/10.1016/j.vetmic.2008.09.012.

Zankari, E., Hasman, H., Kaas, R.S., Seyfarth, A.M., Agersø, Y., Lund, O., Larsen, M.V., Aarestrup, F.M., 2013. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. J Antimicrob Chemother. 68, 771-777. https://doi.org/10.1093/jac/dks496.

Zhang, L., Gao, J., Barkema, H.W., Ali, T., Liu, G., Deng, Y., Naushad, S., Kastelic, J.P., Han, B., 2018. Virulence gene profiles: alpha-hemolysin and clonal diversity in *Staphylococcus aureus* isolates from bovine clinical

mastitis in China. BMC Vet Res. 14, 63. https://doi.org/10.1186/s12917-018-1374-7.

Åvall-Jääskeläinen, S., Taponen, S., Kant, R., Paulin, L., Blom, J., Palva, A., Koort, J., 2018. Comparative genome analysis of 24 bovine-associated *Staphylococcus* isolates with special focus on the putative virulence genes. PeerJ. 6, e4560. https://doi.org/10.7717/peerj.4560.

8. Scientific papers I-III

Paper I

Received: 25 March 2020 | Revised: 21 April 2020 | Accepted: 25 April 2020

DOI: 10.1002/mbo3.1057

ORIGINAL ARTICLE

MicrobiologyOpen Open Access WILEY

Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220

Marte Ekeland Fergestad[1,2] | Gro Anita Stamsås[1] | Danae Morales Angeles[1] | Zhian Salehian[1] | Yngvild Wasteson[2] | Morten Kjos[1]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

[2]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway

Correspondence
Morten Kjos, Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway.
Email: morten.kjos@nmbu.no

Funding information
Research Council of Norway ANIHWA ERA-Net, Grant/Award Number: 258877; Research Council of Norway FRIMEDBIO, Grant/Award Number: 250976; Research Council of Norway JPIAMR, Grant/Award Number: 296906

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to most β-lactams due to the expression of an extra penicillin-binding protein, PBP2a, with low β-lactam affinity. It has long been known that heterologous expression of the PBP2a-encoding *mecA* gene in methicillin-sensitive *S. aureus* (MSSA) provides protection towards β-lactams, however, some reports suggest that the degree of protection can vary between different β-lactams. To test this more systematically, we introduced an IPTG-inducible *mecA* into the MSSA laboratory strain RN4220. We confirm, by growth assays as well as single-cell microfluidics time-lapse microscopy experiments, that PBP2a expression protects against β-lactams in *S. aureus* RN4220. By testing a panel of ten different β-lactams, we conclude that there is also a great variation in the level of protection conferred by PBP2a. Expression of PBP2a resulted in an only fourfold increase in minimum inhibitory concentration (MIC) for imipenem, while a 32-fold increase in MIC was observed for cefaclor and cephalexin. Interestingly, in our experimental setup, PBP2a confers the highest protection against cefaclor and cephalexin—two β-lactams that are known to have a high specific affinity toward the transpeptidase PBP3 of *S. aureus*. Notably, using a single-cell microfluidics setup we demonstrate a considerable phenotypic variation between cells upon β-lactam exposure and show that *mecA*-expressing *S. aureus* can survive β-lactam concentrations much higher than the minimal inhibitory concentrations. We discuss possible explanations and implications of these results including important aspects regarding treatment of infection.

KEYWORDS

mecA, microfluidics, MRSA, time-lapse microscopy, β-lactams

Marte Ekeland Fergestad and Gro Anita Stamsås contributed equally to this work and are listed with increasing seniority.

1 | INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for a range of different infections in both animals and humans, including skin and wound infections, mastitis, and bacteremia. Besides, both animals and humans can be asymptomatic carriers of these bacteria. Traditionally, β-lactams alone or in combination with other substances have been used successfully to treat staphylococcal infections, due to their low toxicity, good pharmacodynamics, and bactericidal action (Foster, 2019; Llarrull, Fisher, & Mobashery, 2009). However, the spread of β-lactam resistant staphylococcal strains has emerged as a global concern (Grundmann, Aires-de-Sousa, Boyce, & Tiemersma, 2006), making it increasingly difficult to combat these infections.

β-lactam antibiotics function by inhibiting the transpeptidase activity of penicillin-binding proteins (PBPs). PBPs are essential for the last steps of the synthesis of peptidoglycan in the bacterial cell wall. Peptidoglycan consists of glycan chains with alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) units, which are cross-linked by peptide bridges between the stem peptides of the NAMs units (Egan, Cleverley, Peters, Lewis, & Vollmer, 2017; Typas, Banzhaf, Gross, & Vollmer, 2011). Synthesis of peptidoglycan requires two enzymatic reactions: NAGs and NAMs are added to the growing peptidoglycan chain by transglycosylases, and the cross-links are formed by transpeptidases (Lovering, Safadi, & Strynadka, 2012; Typas et al., 2011). β-lactams mimic the D-ala-D-ala residues on the NAM side chain and form a covalent bond to a serine residue in the transpeptidase active site to inhibit PBP activity (Peacock & Paterson, 2015).

Staphylococcus aureus encodes four different PBPs, named PBP1-4 (Pinho, Kjos, & Veening, 2013) which can be targeted by β-lactams. Two of these, PBP1 and PBP3, are monofunctional transpeptidases, meaning that they only catalyze the formation of peptide crossbridges. PBP1 and PBP3 interact with proteins of the SEDS (shape, elongation, division, and sporulation) family (FtsW and RodA, respectively) to form active transpeptidase/transglycosylase pairs (Meeske et al., 2016; Reichmann et al., 2019). In contrast to PBP1 and PBP3, the PBP2 is a bifunctional protein with both transpeptidase and transglycosylase activities in the same protein. The last PBP, PBP4 is a nonessential low-molecular-weight PBP with transpeptidase activity, whose function is still to a large extent undefined (da Costa, de Oliveira, Chambers, & Chatterjee, 2018).

Methicillin-resistant *S. aureus* (MRSA) strains encode, in addition to PBP1-4, a fifth PBP protein, known as PBP2a, which is responsible for the resistant phenotype. Even though methicillin is no longer in use, the term methicillin resistance persists and represents resistance to practically all β-lactams, except 5th generation cephalosporins (Peacock & Paterson, 2015). PBP2a is encoded by the *mecA* gene located on a genomic island known as staphylococcal cassette chromosome mec (SCCmec) (Katayama, Ito, & Hiramatsu, 2000). PBP2a is a transpeptidase with a reduced affinity for transpeptidase-inhibiting β-lactams. This low affinity allows MRSA strains to continue cell wall synthesis and multiplication in the presence of β-lactams, as the transpeptidase activity of PBP2a is still functional when the activities of the other PBPs are inhibited.

PBP2a activity in MRSA is regulated on many levels. For example, PBP2a is under allosteric control (Fuda et al., 2005; Otero et al., 2013). Correct folding and activity of PBP2a are also known to be dependent on extracellular chaperones (Jousselin et al., 2015; Roch et al., 2019), while *mecA* transcription is influenced by several factors (Hao, Dai, Wang, Huang, & Yuan, 2012; Peacock & Paterson, 2015). Importantly, the stringent stress response pathway, specifically mediated by changes in the guanine metabolism, is associated with high-level β-lactam resistance in MRSA strains (Kim et al., 2013; Mwangi et al., 2013; Tomasz, Nachman, & Leaf, 1991). Many MRSA strains also display so-called heterogeneous resistance where only a fraction of the cells in a population are resistant (de Lencastre & Tomasz, 1994; Tomasz et al., 1991). Induction of the stringent stress response can change this heterogeneous resistant phenotype to a homogeneous, high-level β-lactam resistant phenotype (Aedo & Tomasz, 2016).

Different β-lactam subclasses, such as penicillins, cephalosporins, carbapenems, and monobactams, all have the β-lactam ring as the functional core. Apart from that, they contain chemical features which give different properties, such as different sensitivities toward β-lactamases (Bush, 2018) and selective affinities for different PBPs (Chambers, Sachdeva, & Kennedy, 1990; Georgopapadakou, Smith, & Bonner, 1982; Kocaoglu, Tsui, Winkler, & Carlson, 2015). Such detailed knowledge about the characteristics of these antibiotics and their interplay with bacteria could be utilized and explored in the design of individually tailored treatment schemes of difficult-to-treat infections. Due to the increasing spread and treatment challenges of MRSA, it is necessary to gain further insight into the β-lactam resistance of *S. aureus*. In this work, we investigated how PBP2a protected against different β-lactams in when expressed in the MSSA-strain *S. aureus* RN4220.

2 | RESULTS AND DISCUSSION

2.1 | Heterologous expression of *mecA* in *S. aureus* RN4220 results in dose-dependent cefoxitin resistance

Heterologous expression of *mecA* has previously been shown to confer resistance to β-lactams in *S. aureus* MSSA strains (Ballhausen, Kriegeskorte, Schleimer, Peters, & Becker, 2014; Matthews, Reed, & Stewart, 1987; Murakami & Tomasz, 1989). We introduced the PBP2a-encoding gene *mecA* downstream of the P_{spac} promoter on a plasmid in the MSSA laboratory strain RN4220 (pLOW-*mecA*, strain MF7). This strain allows controlled *mecA* expression from the well-established pLOW plasmid (Liew et al., 2011) by the addition of increasing concentrations of IPTG. Expression of *mecA* did not influence the growth of the resulting strain; no growth defect was observed in MF7 (P_{spac}-*mecA*) compared to the control strain (vector control strain IM55 carrying pLOW without *mecA*) for any of the

inducer concentrations (Figure 1a). Ender, McCallum, Adhikari, and Berger-Bächi (2004) found that transformation of a type I SCCmec element into a naïve susceptible strain resulted in a slower growth rate compared to the parental strain. However, with our experimental conditions, there was no apparent fitness cost related to heterologous expression of the *mecA* gene in an MSSA.

To first establish that *mecA* expression could confer resistance in RN4220 under our experimental conditions, we exposed the cells to cefoxitin, a cephalosporin commonly used to detect MRSA strains (Skov et al., 2006, 2014). As expected, *mecA* induction protected *S. aureus* against cefoxitin (Figure 1b–f). The MF7 strain (P_{spac}-*mecA*) grown with a range of inducer concentrations (0–1,000 μM IPTG) was exposed to twofold dilution series of cefoxitin to determine the minimum inhibitory concentrations (MIC). The MIC of the uninduced strain was 1 μg/ml (Table 1), and 50 μM IPTG was needed to increase the MIC to cefoxitin twofold (Figure 1b,c). By further increasing the inducer concentration, a maximum of eightfold increase in MIC was obtained compared to the noninduced MF7 strain (Figure 1d,e, Table 1). The MIC of the noninduced MF7-strain was similar to that of the vector control strain (IM55) and the wild-type RN4220, verifying that leakiness of the P_{spac} promoter did not influence the level of resistance (Table 2). We also performed a population analysis profile (Tomasz et al., 1991) of the MF7-strain and control strain toward cefoxitin, by plating the strain onto different concentrations of cefoxitin. The population analysis profile shows a heterogeneous resistant pattern for MF7 (Figure 1g). This result thus suggests that the majority of cells in the population are sensitive to cefoxitin despite expressing *mecA*. This is in line with what has been reported for the heterologous expression of *mecA* before (Katayama, Zhang, Hong, & Chambers, 2003).

To verify that the enzymatic activity of PBP2a is needed for the observed protection, we created a *mecA* mutant construct, in which the active site serine was changed to alanine (S403A). This mutation has previously been shown to abolish the enzymatic activity of PBP2a (Sun, Bauer, & Lu, 1998). As expected, the *mecA*(S403A) mutation (strain MF21) fully abolished the protection by PBP2a in

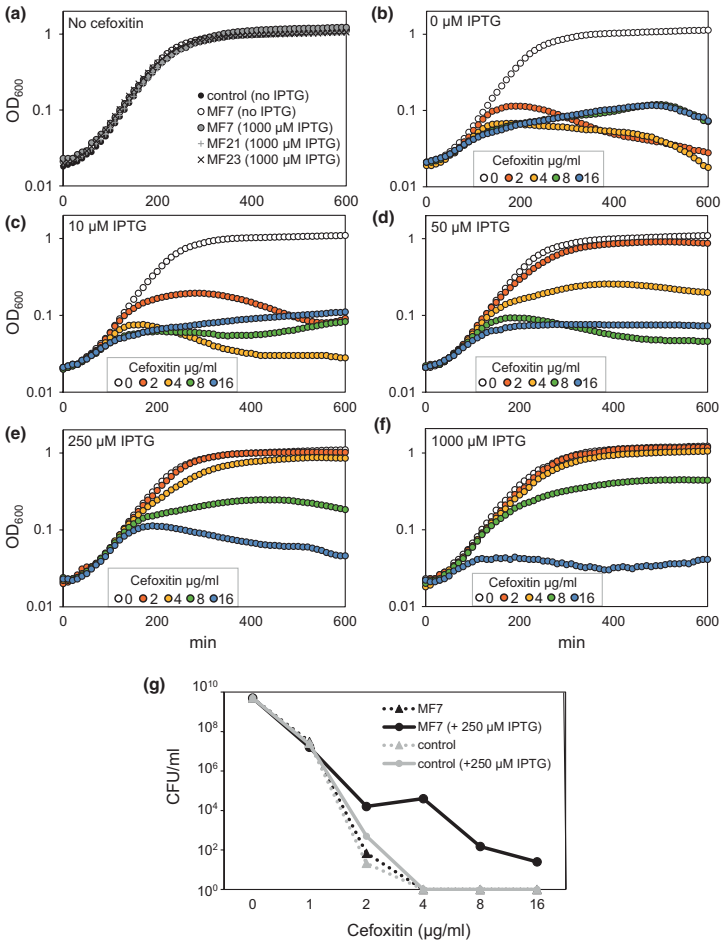


FIGURE 1 Growth of *Staphylococcus aureus* RN4220 expressing *mecA*. (a) Expression of *mecA*, *mecA*(S403A) or *mecA*(K188A) in *S. aureus* does not affect growth. Growth of MF7 (P_{spac}-*mecA*), MF21 (P_{spac}-*mecA*(S403A)), and MF23 (P_{spac}-*mecA*(K188A)) with maximum *mecA* induction (1,000 μM IPTG) were compared to growth of uninduced MF7 and a vector control strain. (b–f) Growth curves of MF7 showing the effect of cefoxitin on growth with different *mecA* induction levels. For each inducer concentration, growth curves in the presence of five different cefoxitin concentrations are shown (0, 2, 4, 8, 16 μg/ml). (b) No *mecA* induction, (c) 10 μM IPTG, (d) 50 μM IPTG, (e) 250 μM IPTG, and (f) 1,000 μM IPTG. (g) Population analysis profile of cefoxitin for MF7 (P_{spac}-*mecA*) and control strain induced with 250 μM IPTG. The strains were plated onto plates with different concentrations of cefoxitin. The population analysis profile shows that MF7 has a heterogeneous resistant phenotype

TABLE 1 Level of protection by *mecA* expression in *Staphylococcus aureus* MF7 for different β-lactams[a]

β-lactam[b]	Class	MIC RN4220 (μg/ml)	MIC MF7 (μg/ml)		Fold protection[c]
			No IPTG	1,000 μM IPTG	
Ampicillin	3rd generation penicillin (extended spectrum)	0.78	0.78	12.5	16
Cefaclor	2nd generation cephalosporin	0.5	0.5	16	32
Cefotaxime	3rd generation cephalosporin	0.5	0.5	4	8
Cefoxitin	2nd generation cephalosporin	1	1	8	8
Cephalexin	1st generation cephalosporin	2	2	64	32
Imipenem	Carbapenem	0.03	0.03	0.13	4
Oxacillin	2nd generation penicillin (narrow spectrum)	0.39	0.39	6.25	16
Penicillin G	1st generation penicillin (narrow spectrum)	0.10	0.10	0.78	8
Piperacillin	4th generation penicillin (extended spectrum)	1.56	1.56	12.5	8

[a]The experiments were repeated at least three times with similar results.

[b]Among the β-lactams tested here, four have been reported to have a specific affinity for certain PBPs in *S. aureus*. These are cefaclor (specific to PBP3), cefotaxime (PBP2), cefoxitin (PBP4), and cephalexin (PBP3).

[c]Fold protection by *mecA* induction was determined as the ratio between MIC with induction and the MIC of uninduced cells.

our experiments (Table 2). Thus, heterologous expression of PBP2a confers protection toward cefoxitin in *S. aureus* RN4220.

PBP2a has also been shown to be under allosteric control (Fuda et al., 2005; Otero et al., 2013), however, it has not been studied whether the allostery of PBP2a plays any role during heterologous expression in an MSSA strain. To see if allosteric regulation played a role under our experimental conditions, we therefore created another mutant, *mecA*(K188A) (strain MF23), where one of the key residues for allosteric regulation was mutated (Otero et al., 2013). However, this mutation did not have any effect on the activity of PBP2a and the mutant was as efficient as the wild-type PBP2a protein in protecting against the different β-lactams (Table 2). Thus, based on the results of this mutant, allosteric regulation does not seem to play any role in our experimental setup.

2.2 | Variable levels of protection toward different β-lactams by PBP2a in *S. aureus* RN4220

The results above establish that the P_{spac}-*mecA* construct in *S. aureus* RN4220 can protect the cells against cefoxitin up to eightfold compared to the controls. Previously, studies have indicated that expression of *mecA* in an MSSA background may confer variable levels of protection against different β-lactams (Ballhausen et al., 2014; Ubukata, Nonoguchi, Matsuhashi, & Konno, 1989). To study this variation more systematically, we tested the resistance levels of strain MF7 toward a panel of 10 different β-lactams. These represent ten different β-lactam subclasses, including penicillins (1st–4th generation), carbapenems, and cephalosporins (1st–3rd generation) (Table 1). Furthermore, it has long been known that different β-lactams have variable affinities for the four native staphylococcal PBPs (Chambers & Sachdeva, 1990; Chambers et al., 1990; Georgopapadakou, Dix, & Mauriz, 1986; Georgopapadakou, Smith, Cimarusti, & Sykes, 1983), and we included β-lactams with variable affinity characteristics: Cefoxitin has the highest affinity for PBP4, cefotaxime has the highest affinity for PBP2 and cephalexin and cefaclor has the highest affinity for PBP3 (Chambers et al., 1990; Georgopapadakou et al., 1982, 1986). The other β-lactams tested also have variable but less defined affinities for PBP1-PBP4 (Chambers & Sachdeva, 1990; Georgopapadakou et al., 1982, 1983, 1986).

MICs were determined in strain MF7 with or without induction of *mecA* by IPTG (Table 1). Notably, the level of protection for the different β-lactams ranged from fourfold to a 32-fold increase in MIC from induced to uninduced. Cefaclor and cephalexin showed the highest increase in MIC (32-fold), followed by ampicillin and oxacillin (16-fold). The lowest MIC increase was found for imipenem (fourfold) followed by cefotaxime, cefoxitin, penicillin G, and piperacillin (all eightfold). These results strengthen and underline previous indications (Ballhausen et al., 2014; Ubukata et al., 1989) that there is a great variation in the level of β-lactam protection conferred by *mecA* upon expression in an *S. aureus* MSSA strain. The observed variations do not seem to correlate with β-lactam subclasses (i.e., penicillins vs cephalosporins, Table 1). The highest level (32-fold) of protection was found against cefaclor and cephalexin. Notably, these are β-lactams that are characterized by having high specific affinity toward PBP3 (Chambers & Sachdeva, 1990; Georgopapadakou et al., 1982). On the other hand, the two other selective β-lactams tested, cefotaxime and cefoxitin, which have the highest affinity toward PBP2 and PBP4, respectively (Chambers & Sachdeva, 1990), showed clearly lower resistance levels (both eightfold resistance).

To further study the notable observation that the highest level of protection was conferred against PBP3-selective β-lactams in our

TABLE 2 MIC values and level of protection against antibiotics with PBP selectivity with a gradual increase in *mecA* expression in *Staphylococcus aureus* MF7[a]

ABX	Strain	Genotype	Concentration IPTG (μM) 0	50	250	1,000	Fold protection[b]
Cefoxitin	IM55	Control	1	1	1	1	1
	MF7	P_{spac}-*mecA*	1	2	4	8	8
	MF21	P_{spac}-*mecA* (S403A)	1	1	1	1	1
	MF23	P_{spac}-*mecA* (K188A)	1	2	8	8	8
Cefotaxime	IM55	Control	0.5	0.5	0.5	0.5	1
	MF7	P_{spac}-*mecA*	0.5	2	4	4	8
	MF21	P_{spac}-*mecA* (S403A)	0.5	0.5	0.5	0.5	1
	MF23	P_{spac}-*mecA* (K188A)	0.5	1	4	4	8
Cefaclor	IM55	Control	0.5	0.5	0.5	0.5	1
	MF7	P_{spac}-*mecA*	0.5	4	16	16	32
	MF21	P_{spac}-*mecA* (S403A)	0.5	0.5	0.5	0.5	1
	MF23	P_{spac}-*mecA* (K188A)	1	4	16	16	32
Cephalexin	IM55	Control	2	2	2	2	1
	MF7	P_{spac}-*mecA*	2	8	64	64	32
	MF21	P_{spac}-*mecA* (S403A)	2	2	2	2	1
	MF23	P_{spac}-*mecA* (K188A)	2	16	32	64	32

[a]The experiments were repeated at least three times with similar results.

[b]Fold protection by *mecA* induction was determined as the ratio between MIC with 1,000 μM induction and the MIC of uninduced cells.

experiments, we made an IPTG titration with concentrations from 0 to 1,000 μM and again determined the MICs for these PBP-selective β-lactams (Table 2). This further demonstrated that PBP2a is more effective in protecting against cefaclor and cephalexin than against cefoxitin and cefotaxime (Table 2) at different PBP2a expression levels. In all cases, the MIC value of the uninduced MF7 was similar to the wild type and control strain, verifying the leaky expression of *mecA* did not play any significant role (Table 1, Table 2). In line with what was found for cefoxitin, the active site mutant did not confer resistance to any of the β-lactams and we found no indication that allosteric regulation plays a role in these assays since the *mecA*(K188A) mutant provided a similar degree of protection as the original *mecA* allele.

PBP3 is a monofunctional, nonessential transpeptidase that is thought to be important for the slight cell elongation observed during the staphylococcal cell cycle (Reichmann et al., 2019). PBP2a, which is also a transpeptidase, replaces the transpeptidase activity of PBP2 in MRSA strains, but cannot complement the transpeptidase activity of PBP1 (Pereira, Henriques, Pinho, de Lencastre, & Tomasz, 2007; Pinho, de Lencastre, & Tomasz, 2001). It is possible that PBP2a functionally complements the β-lactam-inhibited PBP3 activity and that this somehow contributes to the observation that PBP2a expression confers the highest level of resistance toward PBP3-selective β-lactams. This trend has not been reported before. In contrast, Antignac and Tomasz (2009) compared the MICs of the homogeneously resistant MRSA strain COL with an isogenic strain in which *mecA* was deleted (COL-S) and found that the drop in resistance levels was more pronounced for PBP2- and PBP4-specific β-lactams as compared to a PBP3-specific β-lactam (Antignac & Tomasz, 2009; Georgopapadakou et al., 1982). It can be speculated that these differences may be due to strain-specific host factors important for optimal resistance (Berger-Bächi & Rohrer, 2002; Roemer, Schneider, & Pinho, 2013), for example, proteins affecting the cell wall synthesis machinery. However, to shed further light on the mechanisms underlying the observed variation in PBP2a-mediated protection against different β-lactams in *S. aureus* RN4220 observed here, future studies should systematically compare *mecA* expression in *S. aureus* MSSA strains with different genetic backgrounds.

2.3 | Heterologous *mecA* expression confers a low level of protection against β-lactams

In addition to the β-lactam-dependent variation, the results presented in Table 1 also show that the level of protection conferred by *mecA* expression in *S. aureus* RN4220 is relatively low. Upon full induction of *mecA*, the MICs for cefoxitin and cephalexin were 8 and 64 μg/ml, respectively. Both these MICs are significantly lower than those for MRSA strains carrying the full SCCmec. We determined the MICs for cefoxitin and cephalexin for the homogeneous resistant MRSA strain *S. aureus* COL and found these to be 188 and 125 μg/ml, respectively. The relatively low MICs in RN4220 upon heterologous *mecA* expression is in line with a study by Ballhausen et al. (2014) where *mecC* and *mecA* expression in RN4220 resulted in cefoxitin resistance levels in the same range as observed here. These differences between MRSA strains and heterologous expression of *mecA*

in MSSA strains may be explained by the variable transcription level of PBP2a between strains. Alternatively, unknown strain-dependent factors may play an important role. For example, the functionality of PBP2a is likely to be better adapted and optimized in their natural MRSA hosts compared to non-native expression in MSSA hosts such as RN4220 (Katayama et al., 2003). This notion may also be one of several reasons for the limited horizontal gene transfer events observed for *mecA* in *S. aureus* (Peacock & Paterson, 2015) since the competitive advantage of strains with newly acquired *mecA* is relatively low upon exposure to high doses of β-lactams.

2.4 | Single-cell analysis of heterologous *mecA* expression

To further observe how heterologous *mecA* expression protects the RN4220 cells toward β-lactams on a single-cell level, we performed microfluidics fluorescence time-lapse microscopy. To allow cocultivation experiments, we created a GFP-positive RN4220 strain by integrating a *gfp* gene on the RN4220 chromosome, following a previously published approach (de Jong, van der Horst, van Strijp, & Nijland, 2017). The P_{spac}-*mecA*(S403A) construct, expressing the nonactive PBP2A, was transformed into the GFP-positive strain to create strain MF27. As expected, the MICs of strain MF27 were shown to be identical to those of MF21 and wild-type RN4220 (data not shown). The single-cell growth of the two strains MF7 (P_{spac}-*mecA*) and MF27 (P_{spac}-*mecA*(S403A), GFP^+) was then studied in culture medium without cefoxitin and in the presence of 2 and 20 μg/ml cefoxitin. These concentrations correspond to twofold and 20-fold higher than the MIC for *S. aureus* RN4220 (Table 1). Expression from the P_{spac} promoter was induced throughout the experiment. In the absence of cefoxitin (Figure 2a), the two strains were both actively multiplying. At the lower cefoxitin concentration (Figure 2b, 2 μg/ml), MF7 was growing normally, while the MF27 strain stopped dividing. In the presence of 20 μg/ml, which is more than 20× MIC of MF27 and more than 2× MIC of MF7, neither of the strains were multiplying (Figure 2c). Thus, these single-cell data are fully in line with the growth curves.

It was interesting to note, however, that only a fraction of the *mecA*-negative cells (MF27) lysed, as observed by loss of GFP-signal upon exposure to cefoxitin. During the 6 hr timeframe of the experiments, exposure to 2× MIC (2 μg/ml cefoxitin) resulted in lysis of 17.3% (N = 243) of the cells. The fraction increased somewhat upon exposure to 20× MIC of cefoxitin (28.1% of cell lost GFP-signal, N = 238). Still, the majority of cells even at this concentration (20× MIC of MF27) did not lyse. Our observations thus show that there are cell-to-cell variations with regard to cell lysis upon cefoxitin exposure.

To see whether this was a cefoxitin-specific phenotype, the same type of experiments was then performed with cefotaxime, a 3rd generation cephalosporin. The MIC of the control strain for cefotaxime is 0.5 μg/ml, and similar to cefoxitin, induction of *mecA* expression resulted in an eightfold increase in MIC (Table 1). During the time-lapse microscopy, cells were exposed to 2 μg/ml (4× MIC of control) and 20 μg/ml (40× MIC of control) for four hours. In these experiments, we changed to cefotaxime-free medium after four hours to study the potential recovery and regrowth of cells after antibiotic exposure. As for cefoxitin, only a small fraction of the control cell (MF27) lysed during four hours (Figure 3, 8.1% and 7.6%, respectively). However, upon changing to normal growth medium after four hours of cefotaxime exposure, the MF27 cells did not regrow. This shows, as expected, that all the cells were killed by the bactericidal β-lactam although only a fraction of the cells lysed. It is well established that β-lactams inhibit the PBP transpeptidase activity, however, the exact mechanism leading to cell killing by β-lactams is still

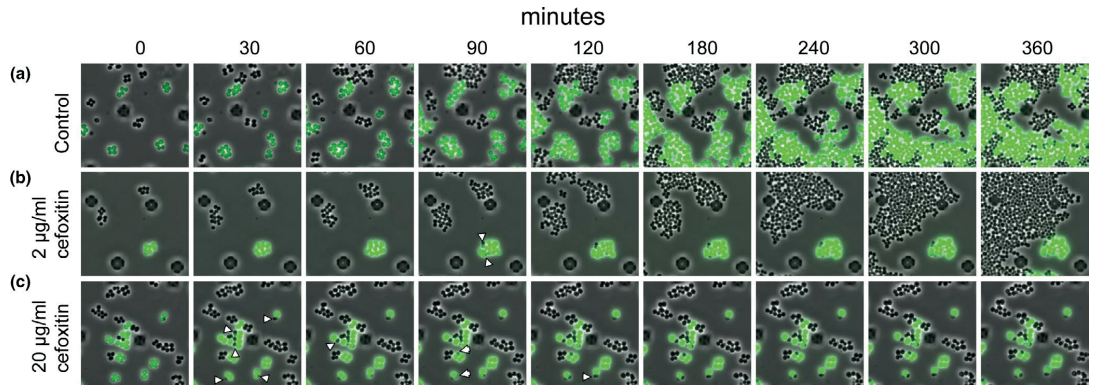


FIGURE 2 Microfluidics fluorescence microscopy time-lapse experiments in the presence of different concentrations of cefoxitin. The two strains MF7 (P_{spac}-*mecA*, dark cells) and MF27 (P_{spac}-*mecA*(S403A)), GFP+ green cells) were mixed in equal ratios, and pregrown in media with 250 μ IPTG to induce expression of the *mecA* alleles. Single-cell growth was analyzed in medium (a) without cefoxitin and in the presence of (b) 2 μg/ml and (c) 20 μg/ml cefoxitin using a CellASIC ONIX Microfluidics setup. 250 μM IPTG was present in all conditions to induce the expression of *mecA* alleles. White arrowheads point to lysing cells. See also Movies S1–S3 (https://doi.org/10.6084/m9.figshare.12168351.v1)

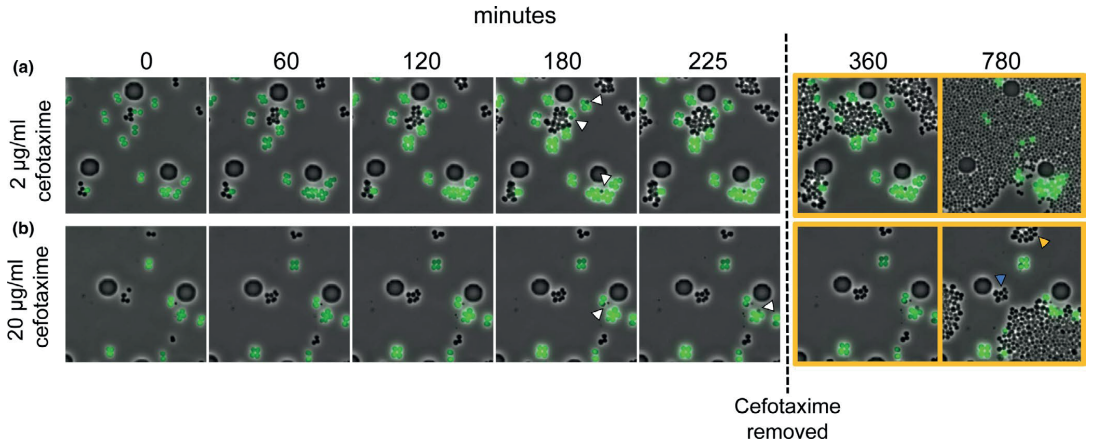


FIGURE 3 Microfluidics fluorescence microscopy time-lapse experiments in the presence of different concentrations of cefotaxime. The two strains MF7 (P_{spac}-*mecA*, dark cells) and MF27 (P_{spac}-*mecA*(S403A)), GFP+ green cells) were mixed in equal ratios, and pregrown in media with 250 µM IPTG to induce expression of the *mecA* alleles. Single-cell growth was analyzed in medium (a) with 2 µg/ml and (c) 20 µg/ml cefotaxime using a CellASIC ONIX Microfluidics setup. 250 µM IPTG was present in all conditions to induce the expression of *mecA* alleles. After 225 min, the cefotaxime-containing medium was changed to regular medium to investigate whether any of the cells could recover. White arrowheads point to lysing cells. Yellow and blue arrowheads point to examples of cell regrowing and not regrowing, respectively, after removal of cefotaxime from the media. See also Movies S4–S5 (https://doi.org/10.6084/m9.figshare.12168351.v1)

not fully understood (Peacock & Paterson, 2015). Cell wall degrading enzymes seem to have variable importance in different strains and for different β-lactams (Peacock & Paterson, 2015). The cell-to-cell variation observed here with regard to lysis thus suggests that several mechanisms resulting in cell killing at play in the same population.

The growth of *mecA*-positive MF7-cells was, as expected, fully inhibited at the highest cefotaxime concentration (20 µg/ml) (Figure 2b). This concentration is fivefold higher than cefotaxime MIC after *mecA* induction (Table 2). Noteworthy, however, upon changing to normal growth medium after four hours of cefotaxime exposure, some of these cells (13%, *N* = 170) were able to regrow after the antibiotic exposure was released (Figure 3, yellow arrowheads). The presence of PBP2a in these cells thus protected the cells from β-lactam-mediated killing even with four hours exposure with concentration much higher than the MIC value. The reason for the cell-to-cell variation is not known. As shown in Figure 1g, the MF7 strain has a heterogeneous resistant phenotype and this result underlines the importance of prolonged drug treatment to kill all cells in a population.

3 | CONCLUSIONS

The results presented here show (a) that the level of resistance conferred by *mecA* expression in *S. aureus* RN4220 is low compared to MRSA strains, and that (b) the level of resistance varies considerably between different β-lactams. Surprisingly, and in contrast to what has been reported for MRSA strains, the highest level of resistance is observed for PBP3-targeting β-lactams. The reason for this is unknown and should be subjected to further studies. It is not known whether these variable MICs observed here would be valid in clinical isolates of MRSA. However, it underlines the importance of determining the MIC for the specific antibiotic toward the individual pathogenic strain when preparing for the treatment of an MRSA infection, as well as considering the achievable drug concentration at the site of infection.

Our experiments were done in a laboratory strain with heterologous expression of *mecA*. If the observations reported here also are representative for wild-type populations of *S. aureus*, this will pose critical problems for diagnostics and treatment of such infections. For example for cefoxitin, the MIC of resistant isolates based on the Clinical and Laboratory Standards Institute (CLSI) guidelines is >8 µg/ml (CLSI, 2020), and wild-type MRSA strains often show MICs more than 10-fold higher (e.g., 188 µg/ml for *S. aureus* COL). Due to the relatively low MIC values after *mecA* induction (e.g., 8 µg/ml for cefoxitin), such a strain could be interpreted as intermediate or even negative for methicillin resistance. Besides, the single-cell data suggest that some cells can survive at concentrations fivefold higher than the MIC and thus also regrow when no longer exposed to antibiotics. This is a critical aspect in the treatment of infections and further highlights the importance of achievable drug concentrations and duration of drug exposure, to prevent such survivor cells to regrow and avoid re-emergence of infections.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains and growth conditions

All strains used in this study are listed in Table 3. *Escherichia coli* was grown in LB medium at 37°C with shaking or on LB plates at 37°C. 100 µg/ml ampicillin was added to the growth medium for selection.

The transformation of *E. coli* was performed using standard heat-shock protocols.

Staphylococcus aureus was grown in brain-heart-infusion (BHI) medium with shaking at 37°C or on BHI agar at 37°C. When appropriate, 5 μg/ml erythromycin was added to the growth medium for selection. Expression from the P_{spac} promoter was induced by the addition of IPTG to the growth medium. Transformation of *S. aureus* was performed by electroporation, as described before (Lofblom, Kronqvist, Uhlen, Stahl, & Wernerus, 2007), with plasmids isolated from *E. coli* IM08B (Monk, Tree, Howden, Stinear, & Foster, 2015).

4.2 | Plasmid and strain construction

All primers and plasmids used in this study are listed in Table 4.

4.2.1 | pLOW-*mecA*

The *mecA* gene was amplified from *S. aureus* COL (Gill et al., 2005) using primers mf3 and mf2. The PCR product was digested with restriction enzymes SalI and EcoRI and ligated into the multiple cloning site of pLOW, to produce the plasmid pLOW-*mecA*, a plasmid with the inducible promoter P_{spac} that allows controlled expression of *mecA*. The ligation reaction was transformed into *E. coli* IM08B, and correct constructs were verified by colony PCR and sequencing with primers IM110 and IM134.

4.2.2 | pLOW-*mecA*(S403A)

The *mecA*(S403A) allele was made by introducing a point mutation using a two-step overlap extension PCR. The first fragment was amplified with primers mf3 and mecA_S403A_r, and the second fragment was amplified with primers mecA_S403A_f and mf2. pLOW-*mecA* was used as template DNA. The mutation was introduced by the overlapping inner primers, and the two fragments were fused in a second PCR using the two outer primers which contain the SalI and EcoRI restriction sites. The final fragment was digested with SalI and EcoRI and ligated into the multiple cloning site of pLOW. The resulting plasmid was verified by PCR and sequencing as described above.

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Escherichia coli		
IM08B		Monk et al. (2015)
Staphylococcus aureus		
RN4220		Kreiswirth et al. (1983)
COL		Gill et al. (2005)
MK1483	RN4220, chromosomal integration of SarA_P1-*sfgfp* in the locus between genes SAOUHSC_00038 and SAOUHSC_00039	This study
MF7	RN4220, pLOW-*mecA*	This study
MF21	RN4220, pLOW-*mecA*(S403A)	This study
MF23	RN4220, pLOW-*mecA*(K188A)	This study
MF27	MK1483, pLOW-*mecA*(S403A)	This study
IM55	RN4220, pLOW-*lacA-gfp*	Lab collection
Plasmids		
pLOW-GFP	Plasmid containing a gfp gene downstream of a P_{spac} promoter (P_{spac}-MCS-*gfp*)	Liew et al. (2011)
pLOW-mecA	Expressing *mecA* from an IPTG-inducible promoter (P_{spac}-*mecA*), ery^R, amp^R	This study
pLOW-mecA(S403A)	Expressing *mecA* with mutation S403A to inactivate the active site P_{spac}-*mecA(S403A)*, ery^R, amp^R	This study
pLOW-mecA(K188A)	Expressing *mecA* with mutation K188A to inactivate the allosteric site P_{spac}-*mecA(K188A)*, ery^R, amp^R	This study
pTH100	Vector for the integration of SarA_P1-sGFP in the locus between genes SAOUHSC_00038 and SAOUHSC_00039 pJB38-NWMN29-30 + SarA_P1-sGFP-Term	de Jong et al. (2017)

TABLE 4 Oligos used in this study

Name	Sequence (5′–3′)
mf3_mecA_f_SalI	ACTGGTCGACGTAATATACTACAAATGTAGTCTT
mf2_mecA_r_EcoRI	GATCGAATTCTCGTTACGGATTGCTTCACTG
im110_seq-pLOW_up ermC	TTGGTTGATAATGAACTGTGCT
im134_pLOW_down_check_R	TGTGCTGCAAGGCGATTAAG
mecA_K188A_f	AGCAATCGCTgcAGAACTAAGTATTTC
mecA_K188A_r	GAAATACTTAGTTCTgcAGCGATTGCT
mecA_S403A_f	ACTTCACCAGGTgCAACTCAAAAAATAT
mecA_S403A_r	ATATTTTTTGAGTTGcACCTGGTGAAGT

4.2.3 | pLOW-*mecA*(K188A)

The *mecA*(K188A) allele was made by the introduction of a point mutation using a two-step overlap extension PCR. The first fragment was amplified with primers mf3 and mecA_K188A_r, and the second fragment was amplified with primers mecA_K188A_f and mf2. pLOW-*mecA* was used as template DNA. The plasmid was then made and verified as described above.

4.2.4 | *Staphylococcus aureus* MK1483

To make a constitutive GFP-positive strain (SarA_P1-*sfgfp* in RN4220), we used the plasmid pTH100, which allows markerless integration of a superfolder gfp expressing construct in an intergenic region between genes SAOUHSC_00038 and SAOUHSC_00039 (de Jong et al., 2017). The temperature-sensitive pTH100 plasmid was transformed into *S. aureus* RN4220 at 30°C using chloramphenicol as a selection marker, and the double crossover was generated as described (de Jong et al., 2017). GFP-positive colonies were finally verified for correct integration by PCR.

4.3 | Growth assays and determination of β-lactam susceptibility

The MICs for different antibiotics were determined by twofold dilution assays in microtiter plates. Overnight cultures of *S. aureus* strains grown in BHI with 5 μg/ml erythromycin were diluted 100-fold in medium (with various IPTG concentrations) and exposed to a twofold dilution series of the antibiotics (listed in Table 1). Growth at 37°C was monitored by measuring OD_{600} every 10th minute for 15 hr in a Synergy (BioTek) or Hidex microtiter plate reader (BioTek) with shaking for 5 s before each measurement. MIC (MIC_{50}) was defined as the minimal concentration to inhibit the growth of at least 50%. The fold protection was determined as the ratio between the MIC value for full *mecA* induction (1,000 μM IPTG) and the MIC value for the uninduced condition. All MIC assays were performed at least three times.

4.4 | Population analysis profile (PAP)

PAPs were performed as described by Reichmann and Pinho (2017) with some modifications. Briefly, overnight cultures of strains MF7 and MF12 with 250 μM IPTG and without IPTG were diluted to 10^{-1} to 10^{-7}. Ten μl of each dilution was plated on BHI plates containing cefoxitin (0, 1, 2, 4, 6, and 16 μg/ml), erythromycin 5 μg/ml and 250 mM IPTG when necessary. Plates were incubated at 37°C, and colonies were counted after 24 hr.

4.5 | Time-lapse microfluidics microscopy

A CellASIC® ONIX2 Microfluidic System (Millipore) connected to a Zeiss fluorescence microscope was used to monitor the growth and survival of *S. aureus* during exposure to cefoxitin and cefotaxime. Strains MF7 and MF27 were grown overnight in BHI with 5 μg/ml erythromycin. The cultures were rediluted in the same medium with 250 μM IPTG for induction and grown for 3 hr until the cultures reached the exponential phase (OD_{600} = 0.4). CellASIC® ONIX B04A-03 Microfluidic Bacteria Plates (Millipore) were primed with medium (BHI with 5 μg/ml erythromycin and 250 μM IPTG), and cells were loaded onto the plates according to the manufacturer's protocol. Images were acquired with a Zeiss Axio Observer with an Orca-Flash4.0 V2 Digital complementary metal-oxide-semiconductor (CMOS) camera (Hamamatsu Photonics) through a 100× PC objective. HPX 120 Illuminator was used as a fluorescent light source. Cells were imaged (phase contrast and GFP fluorescence) every 15th minute for 6 hr during normal growth or exposure to cefoxitin or cefotaxime.

ACKNOWLEDGMENTS

We would like to thank Ine Myrbråten, NMBU, for help with strain construction. This work has been funded by the ANIHWA ERA-Net project MRSA_BACTERIOPHAGE project (project number 258877), by a FRIMEDBIO grant (project number 250976) and a JPIAMR grant (project number 296906) from the Research Council of Norway.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Marte Ekeland Fergestad: Conceptualization (supporting); investigation (lead); methodology (equal); writing – original draft (supporting); writing – review & editing (lead). **Gro Anita Stamsås:** Conceptualization (supporting); investigation (equal); methodology (equal); writing – review & editing (equal). **Danae Morales Angeles:** Investigation (equal); methodology (equal); writing – review & editing (equal). **Zhian Salehian:** Investigation (equal); methodology (equal); writing – review & editing (equal). **Yngvild Wasteson:** Conceptualization (supporting); funding acquisition (equal); supervision (supporting); writing – original draft (equal); writing – review & editing (equal). **Morten Kjos:** Conceptualization (lead); funding acquisition (equal); investigation (supporting); methodology (equal); supervision (lead); writing – original draft (lead); writing – review & editing (lead).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

Data generated in this study are available from the corresponding author upon request. Movies S1–S5 are available at figshare: https://doi.org/10.6084/m9.figshare.12168351.v1.

ORCID

Morten Kjos https://orcid.org/0000-0003-4448-9082

REFERENCES

Aedo, S., & Tomasz, A. (2016). Role of the stringent stress response in the antibiotic resistance phenotype of methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *60*(4), 2311–2317. https://doi.org/10.1128/AAC.02697-15

Antignac, A., & Tomasz, A. (2009). Reconstruction of the phenotypes of methicillin-resistant *Staphylococcus aureus* by replacement of the staphylococcal cassette chromosome mec with a plasmid-borne copy of *Staphylococcus sciuri pbpD* gene. *Antimicrobial Agents and Chemotherapy*, *53*(2), 435–441. https://doi.org/10.1128/AAC.01099-08

Ballhausen, B., Kriegeskorte, A., Schleimer, N., Peters, G., & Becker, K. (2014). The mecA homolog *mecC* confers resistance against β-lactams in *Staphylococcus aureus* irrespective of the genetic strain background. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3791–3798.

Berger-Bächi, B., & Rohrer, S. (2002). Factors influencing methicillin resistance in staphylococci. *Archives of Microbiology*, *178*(3), 165–171. https://doi.org/10.1007/s00203-002-0436-0

Bush, K. (2018). Past and present perspectives on β-lactamases. *Antimicrobial Agents and Chemotherapy*, *62*(10), e01076-01018. https://doi.org/10.1128/AAC.01076-18

Chambers, H. F., & Sachdeva, M. (1990). Binding of β-lactam antibiotics to penicillin-binding proteins in methicillin-resistant *Staphylococcus aureus*. *The Journal of Infectious Diseases*, *161*(6), 1170–1176.

Chambers, H. F., Sachdeva, M., & Kennedy, S. (1990). Binding affinity for penicillin-binding protein 2a correlates with in vivo activity of beta-lactam antibiotics against methicillin-resistant *Staphylococcus aureus*. *Journal of Infectious Diseases*, *162*(3), 705–710.

CLSI (2020). Performance standards for antimicrobial susceptibility testing. In Wayne, P. (Ed.), *CLSI supplement M100* (30th ed.(94). Wayne, PA: Clinical and Laboratory Standards Institute.

da Costa, T. M., de Oliveira, C. R., Chambers, H. F., & Chatterjee, S. S. (2018). PBP4: A new perspective on *Staphylococcus aureus* β-lactam resistance. *Microorganisms*, *6*(3), 57. https://doi.org/10.3390/microorganisms6030057

de Jong, N. W., van der Horst, T., van Strijp, J. A., & Nijland, R. (2017). Fluorescent reporters for markerless genomic integration in *Staphylococcus aureus*. *Scientific Reports*, *7*, 43889. https://doi.org/10.1038/srep43889

de Lencastre, H., & Tomasz, A. (1994). Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *38*(11), 2590–2598. https://doi.org/10.1128/AAC.38.11.2590

Egan, A. J. F., Cleverley, R. M., Peters, K., Lewis, R. J., & Vollmer, W. (2017). Regulation of bacterial cell wall growth. *FEBS Journal*, *284*(6), 851–867. https://doi.org/10.1111/febs.13959

Ender, M., McCallum, N., Adhikari, R., & Berger-Bächi, B. (2004). Fitness cost of SCCmec and methicillin resistance levels in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *48*(6), 2295–2297. https://doi.org/10.1128/AAC.48.6.2295-2297.2004

Foster, T. J. (2019). Can β-lactam antibiotics be resurrected to combat MRSA? *Trends in Microbiology*, *27*(1), 26–38. https://doi.org/10.1016/j.tim.2018.06.005

Fuda, C., Hesek, D., Lee, M., Morio, K., Nowak, T., & Mobashery, S. (2005). Activation for catalysis of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* by bacterial cell wall. *Journal of the American Chemical Society*, *127*(7), 2056–2057.

Georgopapadakou, N. H., Dix, B. A., & Mauriz, Y. R. (1986). Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *29*(2), 333–336. https://doi.org/10.1128/AAC.29.2.333

Georgopapadakou, N. H., Smith, S. A., & Bonner, D. P. (1982). Penicillin-binding proteins in a *Staphylococcus aureus* strain resistant to specific β-lactam antibiotics. *Antimicrobial Agents and Chemotherapy*, *22*(1), 172–175.

Georgopapadakou, N. H., Smith, S. A., Cimarusti, C. M., & Sykes, R. B. (1983). Binding of monobactams to penicillin-binding proteins of *Escherichia coli* and *Staphylococcus aureus*: Relation to antibacterial activity. *Antimicrobial Agents and Chemotherapy*, *23*(1), 98–104. https://doi.org/10.1128/AAC.23.1.98

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., … Fraser, C. M. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology*, *187*(7), 2426–2438. https://doi.org/10.1128/JB.187.7.2426-2438.2005

Grundmann, H., Aires-de-Sousa, M., Boyce, J., & Tiemersma, E. (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. *Lancet (London, England)*, *368*(9538), 874–885. https://doi.org/10.1016/S0140-6736(06)68853-3

Hao, H., Dai, M., Wang, Y., Huang, L., & Yuan, Z. (2012). Key genetic elements and regulation systems in methicillin-resistant *Staphylococcus aureus*. *Future Microbiology*, *7*(11), 1315–1329.

Jousselin, A., Manzano, C., Biette, A., Reed, P., Pinho, M. G., Rosato, A. E., … Renzoni, A. (2015). The *Staphylococcus aureus* chaperone PrsA is a new auxiliary factor of oxacillin resistance affecting penicillin-binding protein 2A. *Antimicrobial Agents and Chemotherapy*, *60*(3), 1656–1666.

Katayama, Y., Ito, T., & Hiramatsu, K. (2000). A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *44*(6), 1549–1555. https://doi.org/10.1128/AAC.44.6.1549-1555.2000

Katayama, Y., Zhang, H. Z., Hong, D., & Chambers, H. F. (2003). Jumping the barrier to beta-lactam resistance in *Staphylococcus aureus*. *Journal of Bacteriology*, *185*(18), 5465–5472.

Kim, C., Mwangi, M., Chung, M., Milheirico, C., de Lencastre, H., & Tomasz, A. (2013). The mechanism of heterogeneous β-lactam resistance in MRSA: Key role of the stringent stress response. *PLoS ONE*, *8*(12), e82814.

Kocaoglu, O., Tsui, H.-C.-T., Winkler, M. E., & Carlson, E. E. (2015). Profiling of β-lactam selectivity for penicillin-binding proteins in *Streptococcus pneumoniae* D39. *Antimicrobial Agents and Chemotherapy*, *59*(6), 3548–3555. https://doi.org/10.1128/AAC.05142-14

Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature*, *305*(5936), 709–712. https://doi.org/10.1038/305709a0

Liew, A. T., Theis, T., Jensen, S. O., Garcia-Lara, J., Foster, S. J., Firth, N., … Harry, E. J. (2011). A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in *Staphylococcus aureus*. *Microbiology*, *157*(Pt 3), 666–676. https://doi.org/10.1099/mic.0.045146-0

Llarrull, L. I., Fisher, J. F., & Mobashery, S. (2009). Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new β-lactams that meet the challenge. *Antimicrobial Agents and Chemotherapy*, *53*(10), 4051–4063.

Lofblom, J., Kronqvist, N., Uhlen, M., Stahl, S., & Wernerus, H. (2007). Optimization of electroporation-mediated transformation: *Staphylococcus carnosus* as model organism. *Journal of Applied Microbiology*, *102*(3), 736–747. https://doi.org/10.1111/j.1365-2672.2006.03127.x

Lovering, A. L., Safadi, S. S., & Strynadka, N. C. J. (2012). Structural perspective of peptidoglycan biosynthesis and assembly. *Annual Review of Biochemistry*, *81*, 451–478. https://doi.org/10.1146/annurev-biochem-061809-112742

Matthews, P. R., Reed, K. C., & Stewart, P. R. (1987). The cloning of chromosomal DNA associated with methicillin and other resistances in *Staphylococcus aureus*. *Journal of General Microbiology*, *133*(7), 1919–1929. https://doi.org/10.1099/00221287-133-7-1919

Meeske, A. J., Riley, E. P., Robins, W. P., Uehara, T., Mekalanos, J. J., Kahne, D., … Rudner, D. Z. (2016). SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature*, *537*(7622), 634–638. https://doi.org/10.1038/nature19331

Monk, I. R., Tree, J. J., Howden, B. P., Stinear, T. P., & Foster, T. J. (2015). Complete bypass of restriction systems for major *Staphylococcus aureus* lineages. *MBio*, *6*(3), e00308. https://doi.org/10.1128/mBio.00308-15

Murakami, K., & Tomasz, A. (1989). Involvement of multiple genetic determinants in high-level methicillin resistance in *Staphylococcus aureus*. *Journal of Bacteriology*, *171*(2), 874–879. https://doi.org/10.1128/JB.171.2.874-879.1989

Mwangi, M. M., Kim, C., Chung, M., Tsai, J., Vijayadamodar, G., Benitez, M., … Tomasz, A. (2013). Whole-genome sequencing reveals a link between β-lactam resistance and synthetases of the alarmone (p)ppGpp in *Staphylococcus aureus*. *Microbial Drug Resistance*, *19*(3), 153–159.

Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrasco-López, C., Kumarasiri, M., Lastochkin, E., … Hermoso, J. A. (2013). How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(42), 16808–16813. https://doi.org/10.1073/pnas.1300118110

Peacock, S. J., & Paterson, G. K. (2015). Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annual Review of Biochemistry*, *84*, 577–601.

Pereira, S. F., Henriques, A. O., Pinho, M. G., de Lencastre, H., & Tomasz, A. (2007). Role of PBP1 in cell division of *Staphylococcus aureus*. *Journal of Bacteriology*, *189*(9), 3525–3531. https://doi.org/10.1128/JB.00044-07

Pinho, M. G., de Lencastre, H., & Tomasz, A. (2001). An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(19), 10886–10891. https://doi.org/10.1073/pnas.191260798

Pinho, M. G., Kjos, M., & Veening, J. W. (2013). How to get (a)round: Mechanisms controlling growth and division of coccoid bacteria. *Nature Reviews Microbiology*, *11*(9), 601–614. https://doi.org/10.1038/nrmicro3088

Reichmann, N. T., & Pinho, M. G. (2017). Role of SCCmec type in resistance to the synergistic activity of oxacillin and cefoxitin in MRSA. *Scientific Reports*, *7*(1), 6154. https://doi.org/10.1038/s41598-017-06329-2

Reichmann, N. T., Tavares, A. C., Saraiva, B. M., Jousselin, A., Reed, P., Pereira, A. R., … Pinho, M. G. (2019). SEDS-bPBP pairs direct lateral and septal peptidoglycan synthesis in *Staphylococcus aureus*. *Nat Microbiol*, *4*(8), 1368–1377. https://doi.org/10.1038/s41564-019-0437-2

Roch, M., Lelong, E., Panasenko, O. O., Sierra, R., Renzoni, A., & Kelley, W. L. (2019). Thermosensitive PBP2a requires extracellular folding factors PrsA and HtrA1 for *Staphylococcus aureus* MRSA β-lactam resistance. *Communications Biology*, *2*, 417–417. https://doi.org/10.1038/s42003-019-0667-0

Roemer, T., Schneider, T., & Pinho, M. G. (2013). Auxiliary factors: A chink in the armor of MRSA resistance to β-lactam antibiotics. *Current Opinion in Microbiology*, *16*(5), 538–548. https://doi.org/10.1016/j.mib.2013.06.012

Skov, R., Larsen, A. R., Kearns, A., Holmes, M., Teale, C., Edwards, G., & Hill, R. (2014). Phenotypic detection of *mecC*-MRSA: Cefoxitin is more reliable than oxacillin. *Journal of Antimicrobial Chemotherapy*, *69*(1), 133–135. https://doi.org/10.1093/jac/dkt341

Skov, R., Smyth, R., Larsen, A. R., Bolmstrôm, A., Karlsson, A., Mills, K., … Kahlmeter, G. (2006). Phenotypic detection of methicillin resistance in *Staphylococcus aureus* by disk diffusion testing and Etest on Mueller-Hinton agar. *Journal of Clinical Microbiology*, *44*(12), 4395–4399. https://doi.org/10.1128/JCM.01411-06

Sun, Y., Bauer, M. D., & Lu, W. (1998). Identification of the active site serine of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* by electrospray mass spectrometry. *Journal of Mass Spectrometry*, *33*(10), 1009–1016. https://doi.org/10.1002/(SICI)1096-9888(1998100)33:10<1009:AID-JMS717>3.0.CO;2-4

Tomasz, A., Nachman, S., & Leaf, H. (1991). Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. *Antimicrobial Agents and Chemotherapy*, *35*(1), 124–129. https://doi.org/10.1128/AAC.35.1.124

Typas, A., Banzhaf, M., Gross, C. A., & Vollmer, W. (2011). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*, *10*(2), 123–136.

Ubukata, K., Nonoguchi, R., Matsuhashi, M., & Konno, M. (1989). Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. *Journal of Bacteriology*, *171*(5), 2882.

How to cite this article: Fergestad ME, Stamsås GA, Morales Angeles D, Salehian Z, Wasteson Y, Kjos M. Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220. *MicrobiologyOpen*. 2020;9:e1057. https://doi.org/10.1002/mbo3.1057

Paper II

J. Dairy Sci. 104
https://doi.org/10.3168/jds.2020-19988

Antimicrobial resistance and virulence characteristics in 3 collections of staphylococci from bovine milk samples

M. E. Fergestad,[1] A. De Visscher,[2]* T. L'Abee-Lund,[1] C. Ngassam Tchamba,[3] J. G. Mainil,[3] D. Thiry,[3] S. De Vliegher,[2] and Y. Wasteson[1]†
[1]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 0454 Oslo, Norway
[2]M-team and Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University (UGent), 9820 Merelbeke, Belgium
[3]Bacteriology, Department of Infection Diseases, Faculty of Veterinary Medicine, Fundamental and Applied Research in Animal and Health (FARAH) Centre, University of Liège (ULiège), 4000 Liège, Belgium

ABSTRACT

Mastitis is a prevalent disease in dairy cattle, and staphylococci are among the most common causative pathogens. Staphylococci can express resistance to a range of antimicrobials, of which methicillin resistance is of particular public health concern. Additionally, *Staphylococcus aureus* carries a variety of virulence factors, although less is understood about the virulence of non-*aureus* staphylococci (NAS). The aim of our study was to identify and characterize 3 collections of staphylococcal isolates from bovine milk samples regarding antimicrobial resistance, with emphasis on methicillin resistance, and their carriage of virulence genes typically displayed by *Staph. aureus*. A total of 272 staphylococcal isolates collected in Norway and Belgium in 2016 were included, distributed as follows: group 1, Norway, 100 isolates; group 2, Flanders, Belgium, 64 isolates; group 3, Wallonia, Belgium, 108 isolates. Species identification was performed by use of MALDI-TOF mass spectrometry. Phenotypic resistance was determined via disk diffusion, and PCR was used for detection of methicillin resistance genes, *mecA* and *mecC*, and virulence genes. Antimicrobial resistance was common in *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* from all different groups, with resistance to trimethoprim-sulfonamide frequently occurring in *Staph. epidermidis* and *Staph. haemolyticus* as well as in *Staph. aureus*. Resistance to penicillin was most frequently observed in group 1. Ten Belgian isolates (1 from group 2, 9 from group 3) carried the methicillin resistance determinant *mecA*: 5 *Staph. aureus* from 2 different farms and 5 NAS from 3 different farms. Almost all *Staph. aureus* isolates were positive for at least 3 of the screened virulence genes, whereas, in total, only 8 NAS isolates harbored any of the same genes. Our study contributes to the continuous need for knowledge regarding staphylococci from food-producing animals as a basis for better understanding of occurrence of resistance and virulence traits in these bacteria.

Key words: *Staphylococcus aureus*, non-*aureus* staphylococci, antimicrobial resistance, virulence genes, bovine mastitis

INTRODUCTION

Mastitis is a common disease in dairy cattle, affecting animal health and welfare, and dairy farm profitability (Halasa et al., 2007; Rollin et al., 2015). Staphylococci are among the most recognized udder pathogens, of which *Staphylococcus aureus* is an important cause of subclinical and clinical mastitis in dairy cattle, with its importance in the context of mastitis and milk quality varying by region and farm (Østerås et al., 2006; Olde Riekerink et al., 2008; Gao et al., 2017). In a Belgian study, *Staph. aureus* was isolated from 7.3% of the clinical mastitis cases in Flemish dairy herds (Verbeke et al., 2014), whereas in a Norwegian study *Staph. aureus* was found in 45.8% of the clinical mastitis cases from heifers (Waage et al., 1999). In contrast, NAS are the most frequently isolated bacteria causing subclinical mastitis. Their role as mastitis-causing pathogens is debatable, but in general they are associated with moderately increased SCC (Piepers et al., 2007; De Vliegher et al., 2012; De Visscher et al., 2016; Valckenier et al., 2019). A negative effect on milk production after infection has been reported (Heikkilä et al., 2018), although others have reported differently (Valckenier et al., 2019). The distribution of NAS species in milk

Received December 2, 2020.
Accepted March 27, 2021.
*Present address: Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology and Food Science, Agricultural Engineering, Burg. Van Gansberghelaan 115 bus 1, 9820 Merelbeke, Belgium
†Corresponding author: yngvild.wasteson@nmbu.no

samples is described in several studies; however, the results are not directly comparable due to a variety of study designs. A recent study from Belgium concluded that the 5 most prevalent NAS species recovered from quarter milk samples were *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus equorum*, *Staphylococcus hominis*, and *Staphylococcus cohnii* (Wuytack et al., 2020). In Norway, *Staphylococcus epidermidis* and *Staph. aureus* are currently regarded as the most common staphylococcal udder pathogens (Dalen et al., 2019).

Most of the antimicrobial usage in dairy production concerns udder health (Saini et al., 2012; Nobrega et al., 2017). The largest proportion is administered for the intramammary treatment of clinical mastitis and dry cow therapy (De Briyne et al., 2014; Stevens et al., 2016; Stevens et al., 2018). In general terms, treatment with antimicrobials is regarded as the main driver for antimicrobial resistance, although the association between antimicrobial use and corresponding resistance is less compelling in the case of mastitis (Tacconelli, 2009; Oliver and Murinda, 2012).

Methicillin-resistant *Staph. aureus* (**MRSA**) is classified as a high-priority pathogen by WHO and has gained most attention among the resistant staphylococci. However, methicillin resistance has also been described in several species of the NAS group (Feßler et al., 2010; Gindonis et al., 2013), and NAS are thought to be a reservoir for numerous resistance genes that could be transferred into the more pathogenic *Staph. aureus* (Becker et al., 2014). Methicillin-resistant staphylococci are resistant to almost all β-lactams, and infections caused by these bacteria result in limited treatment options, delayed initiation of effective treatment, and poorer outcomes (Yaw et al., 2014). When it comes to MRSA as cause of mastitis, Norway may be regarded as a naive country, as MRSA has been associated with bovine mastitis on only 1 occasion (NORM/NORM-VET, 2015). This contrasts with the current situation in Belgium, where MRSA is an established cause of clinical mastitis. Bardiau et al. (2013) found MRSA in 4.4% of milk samples from bovine mastitis, and Vanderhaeghen et al. (2010) found 9.3% of *Staph. aureus* isolates to be MRSA in milk samples from farms experiencing *Staph. aureus* mastitis.

Staphylococci are well known for their carriage of a range of other resistance determinants in addition to the methicillin resistance trait, often occurring in different combinations, giving rise to multidrug-resistant isolates (Kadlec et al., 2012; Wendlandt et al., 2015; Schoenfelder et al., 2017). An important aspect of the antimicrobial resistance challenge is that resistant staphylococci from animals may pose a threat to public health due to the possible transfer from their different reservoirs to humans (Lee, 2003), and the possible transmission and spread of resistance genes between staphylococci (Levy and Marshall, 2004; Hanssen and Ericson Sollid, 2006). VetPath, a pan-European antimicrobial susceptibility monitoring program, has recently reported data on antimicrobial susceptibility of 9 udder pathogens from bovine mastitis in Europe from 2015 to 2016, and on an overall level they report low resistance to antimicrobials and a low prevalence of MRSA (El Garch et al., 2020).

Mastitis-related *Staph. aureus* are also associated with a variety of virulence factors that play an important role in the pathogenesis of mastitis. These include, among others, cell wall–associated factors, different enzymes, and exotoxins that facilitate the infection pathway. The NAS species lack the aggressive potential of *Staph. aureus*, and less is known about the virulence of these species (Becker et al., 2014; Naushad et al., 2019; Wuytack et al., 2020). Knowledge regarding virulence of both *Staph. aureus* and NAS species associated with bovine mastitis, especially in combination with resistance patterns, is important for designing efficient prophylaxis and treatment guidelines.

The aim of this study was to gain knowledge on resistance and virulence characteristics of 3 groups of mastitis-associated *Staph. aureus* and NAS isolates from bovine milk samples collected in Norway and Belgium as part of an international collaboration. The objectives were (1) to characterize the isolates with regard to their antimicrobial resistance properties, with emphasis on methicillin resistance, (2) to determine carriage of genes encoding known *Staph. aureus* virulence factors in all 3 *Staph. aureus* and NAS isolate collections, and (3) to analyze for correlation patterns of resistance and of virulence of the collected isolates.

MATERIALS AND METHODS

Collection of Staphylococcal Isolates

The isolates were collected as 3 unique collections, all being part of a larger European collaboration. The 3 different sampling strategies, ensuring diversity of the material and at the same time conveniently adapted to the national structures of dairy production, the framework, and the resources of the collaborative project, are described in Table 1. Table 1 also contains information about characteristics of the sampled farms. The aim was to collect a total of 300 staphylococcal isolates: 100 from Norway and 200 from Belgium. The ratio between *Staph. aureus* and NAS was defined a priori to be 1:5. All sampling was performed in 2016.

Table 1. Information about number of isolates, farms sampled, sampling strategies, and characteristics of farms for the 3 different collections of staphylococcal isolates studied

Item	No. of *Staphylococcus aureus* isolates	No. of NAS isolates	No. of farms sampled and total number of isolates	Sampling strategy	Housing of animals and milking systems
Group 1, Norway	20	80	100 farms Total number of isolates = 100, i.e., 1 isolate per farm.	Staphylococci were isolated from milk samples submitted to the routine diagnostics of the TINE Norwegian Dairies Mastitis Laboratory. Only *Staph. aureus* isolated in pure culture from milk samples of cows with clinical mastitis were included in the collection. The NAS isolates were collected from samples with rich growth of NAS in pure culture, sent to the laboratory because of high SCC or clinical mastitis.	Not available per farm, but Norwegian dairy production is characterized by varying housing conditions, including tiestall barns (40%) and loose housing with automatic milking system (47%) or milking parlor (13; Mikalsen et al., 2019).
Group 2, Flanders, Belgium	11	53	NAS isolates were collected on 2 farms with 56 and 49 lactating cows at the time of sampling (n = 105), respectively (new sampling). No *Staph. aureus* were found in this new sampling. *Staph. aureus* isolates were collected in 8 different herds (De Visscher et al., 2016). Total number of isolates = 64.	All quarters from lactating cows in 2 herds with a high *Staphylococcus* prevalence, as measured in a previous study (De Visscher et al., 2016), were sampled during one new cross-sectional sampling and quarter SCC (qSCC) were measured. *Staph. aureus* isolates were retrieved from the collection of the Mastitis and Milk Quality Research Unit of Ghent University and qSCC measurements were available.	Cows were housed in freestall barns with slatted floors and cubicles with mattresses. Conventional milking parlor.
Group 3, Wallonia, Belgium	14	94	The 3 herds had 118, 147, and 94 cows at the time of sampling. Total number of isolates = 108.	Three herds with a subclinical mastitis problem were chosen from 300 farms previously included in another project (MammiScan project, Service Public Wallonie, Division Générale de l'Agriculture RNE; A. S. Rao and L. Théron, Faculty of Veterinary Medicine, University of Liège, Belgium, unpublished data). All cows with a cow somatic cell count (cSCC) >300,000 cell/mL on the last DHI sampling were selected (n = 114), and all quarters were sampled.	One herd as freestall barn with straw, 2 herds with slatted floor. Conventional milking parlor.
Total	45	227	272		

Isolation and Identification of Staphylococcus Isolates

First, 10 μL of each quarter milk sample were plated on a quadrant of a Columbia agar supplemented with 5% sheep blood (Oxoid) for samples from group 2 and 3, and Difco heart infusion agar with 5% washed bovine erythrocytes (BD Biosciences) for samples from group 1, both nonselective media. For groups 2 and 3, modified Chapman's agar or mannitol salt agar, a semiselective medium, were also used for the recovery of staphylococci (Oxoid). Plates were examined after 24-h aerobic incubation at 37°C. If more than 2 phenotypically different colony types were present on blood agar, the quarter milk sample was considered contaminated and rejected. If not, for samples from group 2 and 3 all phenotypically different colony types were counted on mannitol salt agar and subcultured (1 colony per colony type) on blood agar (Columbia agar with 5% sheep blood, Oxoid), whereas for group 1 all suspected staphylococcal colonies on blood agar, based on colony morphology and catalase test, were subcultured (1 colony per colony type) on blood agar (Difco heart infusion agar with 5% washed bovine erythrocytes, BD Biosciences) to obtain pure cultures (aerobic incubation at 37°C, a half plate per colony). The previously mentioned procedures were performed at the laboratories at the place of collection (TINE Norwegian Dairies Mastitis Laboratory, Ghent University, and University of Liège). Pure cultures were examined as described by De Visscher et al. (2013). All suspected staphylococci were stored at −80°C in Microbank cryovials (Pro-Lab Diagnostics) and identified at the species level using MALDI-TOF MS and a validated and updated library for bovine-related NAS species (Cameron et al., 2017, 2018). If no identification could be assigned using MALDI-TOF MS, DNA was subjected to sequencing of the *rpoB* gene (Supré et al., 2009). The examination of pure cultures and species identification with MALDI-TOF MS (or the *rpoB* gene) for all groups were performed at Ghent University.

DNA Extraction

Bacterial DNA was extracted using the following procedure, based on Unal et al. (1992): 1 or 2 colonies of each *Staphylococcus* isolate were mixed with 3 mL of brain heart infusion broth (Oxoid) and aerobically incubated at 37°C overnight. Then 100 μL of each bacterial suspension was centrifuged at 16,500 × *g* for 3 min (Thermo Scientific Heraeus Pico 21 microcentrifuge). The supernatant was removed, and the pellet was resolved with 50 μL of lysostaphin (50 μg/mL). The tubes were heated at 37°C for 10 min before adding 50 μL of proteinase K (100 μg/mL) and 150 μL of 0.1 *M* Tris (pH 7.5), and then heated at 37°C for 10 min and 95°C for 5 min. The extracted DNA was stored at −20°C before analyses.

Phenotypic Antimicrobial Resistance Testing

Antimicrobial resistance was determined by means of the disk diffusion method by Bauer et al. (1966) and according to the methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org, accessed Aug. 2018), except that Mueller Hinton broth was used instead of saline water for the bacterial suspension, corresponding to 0.5 McFarland for the panel of antimicrobial agents. The following antimicrobial agents were included in the test panel: ampicillin (10 μg), amoxicillin and clavulanic acid (20 + 10 μg), ciprofloxacin (5 μg), clindamycin (10 μg), erythromycin (15 μg), gentamicin (10 μg), linezolide (10 μg), penicillin (1 U), trimethoprim (5 μg), sulfonamide and trimethoprim (19:1, 25 μg), and tetracycline (30 μg). Cefoxitin (30 μg) was used for determination of phenotypic methicillin resistance, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Categorization of the isolates as resistant or intermediate (assigned as resistant) versus susceptible was based on clinical breakpoints determined by EUCAST; these breakpoints are prepared for human strains and not mastitis pathogens. Species-specific breakpoints for the NAS group are scarce, so the general breakpoints for coagulase-negative staphylococci were used.

Genotypic β-Lactam Resistance Testing

The PCR for detection of *mecA* and *mecC* was carried out as described by Stegger et al. (2012) to test for β-lactam resistance (methicillin resistance). Genes, primer sequences, amplicon sizes, and annealing temperatures are listed in Table 2. A *mecA*-positive *Staph. aureus* isolate previously confirmed using DNA sequencing (NMBU2664/16, Routine Microbiology Laboratory, Faculty of Veterinary Medicine, Norwegian University of Life Science) and *Staph. aureus* NTCT 13552 were included as positive controls in the *mecA* and *mecC* PCR, respectively. Sterile water was used as negative control.

Virulence Genes

Nine virulence genes common in *Staph. aureus* were selected for PCR analysis, based on a review of relevant articles available in the PubMed database in November and December 2016. The chosen genes were *clfA* (clumping factor), *fnbpA* and *fnbpB* (fibronectin-bind-

Table 2. Genes, proteins, primer sequences, amplicon sizes, and annealing temperatures for all virulence and resistance genes analyzed in the collection of 272 staphylococcal isolates

Gene, protein	Primer name	Primer sequence	Amplicon size	Annealing temperature	Source
hla	hla-F	GGT TTA GCC TGG CCT TC	534	57°C	Wang et al., 2016
α-Hemolysin	hla-R	CAT CAC GAA CTC GTT CG			
hlb	hlb-F	GCC AAA GCC GAA TCT AAG	833	57.5°C	Wang et al., 2016
β-Hemolysin	hlb-R	CGC ATA TAC ATC CCA TGG C			
fnbpA	fnbpA-F	GCG GAG ATC AAA GAC AA	1,279	48°C	Wang et al., 2016
Fibronectin-binding protein A	fnbpA-R	CAT CTA TAG CTG TGT GG			
fnbpB	fnbpB-F	GGA GAA GGA ATT AAG GCG	812	45°C	Wang et al., 2016
Fibronectin-binding protein B	fnbpB-R	GCC GTC GCC TTG AGC GT			
tsst01	tsst-1-F	GCT TGC GAC AAC TGC TAC AG	599	61°C	Wang et al., 2016
Toxic shock syndrome toxin-1	tsst-1-R	TGG ATC CGT CAT TCA TTG TTA T			
ssl7 (*set1*)	ssl7-F	GGT TTA TTC ATA GCG CAG TAT C	879	58°C	Salasia et al., 2004
Staphylococcal superantigen-like protein	ssl7-R	CAA CGT TTC ATC GTT AAG CTG C			
clfA	clfA-F	GGC AAC GAA TCA AGC TAA TAC AC	719	58°C	Wang et al., 2016
Clumping factor A	clfA-R	TTG TAC TAC CTA TGC CAG TTG TC			
mecA[1]	mecA-P4	TCC AGA TTA CAA CTT CAC CAG G	162	55°C	Stegger et al., 2012
Penicillin-binding protein 2a	mecA-P7	CCA CTT CAT ATC TTG TAA CG			
mecC[1]	mecC-F	GAA AAA AAG GCT TAG AAC GCC TC	138	55°C	Stegger et al., 2012
Penicillin-binding protein 2a	mecC-R	GAA GAT CTT TTC CGT TTT CAG C			
cap5	cap5-H	ATG AGG ATA GCG ATT GAA AA	518	49.7°C	Ote et al., 2011
Capsular polysaccharide 5 synthesis enzyme	cap5-R	CGC TTC TTA ATC ACT TTT GC			
cap8	cap8-H	ATC GAA GAA CAT ATC CAA GG	834	46.4°C	Ote et al., 2011
Capsular polysaccharide 8 synthesis enzyme	cap8-R	TTC ATC ACC AAT ACC TTT TA			

[1]Multiplex PCR.

ing proteins), *hla* and *hlb* (hemolysins), *tsst01* (toxic shock syndrome toxin-1), *ssl7* (*set1*, staphylococcal superantigen-like protein), *cap5* (CP5 capsule synthesis enzyme), and *cap8* (CP8 capsule synthesis enzyme; Salasia et al., 2004; Ote et al., 2011; Wang et al., 2016). The PCR were carried out using the protocol of Wang et al. (2016) for *clfA*, *fnbpA*, *fnbpB*, *hla*, *hlb*, and *tsst01*, according to Salasia et al. (2004) for *ssl7*, and according to Ote et al. (2011) for *cap5* and *cap8*.

Genes, primer sequences, amplicon sizes, and annealing temperatures are listed in Table 2. Because positive controls were not available for any of the virulence genes except *cap5* and *cap8*, the amplified PCR products from 1 isolate positive for all the remaining tested virulence genes was confirmed by DNA sequencing, using Sanger sequencing (GATC, Eurofins Genomics) of each gene, and the DNA from the confirmed isolate was further used as positive control. For *cap5* and *cap8*, previously published positive strains B34 and B79 were used as positive controls (Ote et al., 2011). Sterile water was used as negative control.

Correlation Plots

Correlation plots were created in R 3.6.2 (R Core Team, 2013) using the corrplot (Wei and Simko, 2017) package v. 0.84. We considered 2 variables with a phi coefficient greater than 0.7 to be strongly correlated. The correlations that were calculated were correlations between resistance genes in *Staph. aureus*, between resistance genes in NAS, and between virulence genes in *Staph. aureus*. Correlation between virulence genes in NAS was not calculated, due to the low number of virulence genes detected.

RESULTS

Staphylococcus Species Identification

A total of 272 *Staphylococcus* isolates from 319 cattle, collected in Norway (group 1, 100 isolates/100 cattle), Flanders (group 2, 64 isolates/105 cattle), and Wallonia (group 3, 108 isolates/114 cattle), were collected, and all were included in the study. After analysis with MALDI-TOF MS, the following *Staphylococcus* species were most frequently (≥10%) identified: in group 1 *Staphylococcus simulans* (n = 29), *Staph. chromogenes* (n = 25), *Staph. aureus* (n = 20), and *Staph. epidermidis* (n = 13); in group 2: *Staph. chromogenes* (n = 15), *Staph. haemolyticus* (n = 11), *Staph. aureus* (n = 11), and *Staph. cohnii* (n = 8); and in group 3: *Staph. epidermidis* (n = 19), *Staph. chromogenes* (n = 17), *Staph. haemolyticus* (n = 15), and *Staph. aureus* (n = 14). The full species distribution in relation to each group is shown in Figure 1.

Phenotypic Antimicrobial Resistance

Species distribution and number of isolates categorized as resistant toward the panel of antimicrobials tested are shown in Table 3. Antimicrobial resistance was most common in *Staph. haemolyticus* and *Staph. epidermidis* in all 3 sample groups.

Group 1. All 3 *Staph. haemolyticus* isolates and 77% of the *Staph. epidermidis* isolates (n = 10) from group 1 showed resistance to at least 1 of the tested antimicrobials. Resistance to penicillin was more frequently observed, except in the *Staph. simulans* isolates, in which resistance to trimethoprim was most common. *Staphylococcus epidermidis* was the only species of which more than 50% of the isolates were resistant, as 54% were resistant to penicillin. However, no resistance to cefoxitin was observed in this collection. Neither were any isolates categorized as multidrug resistant, defined as resistance to 3 or more of the tested classes of antimicrobials. Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. chromogenes* 17/25, *Staph. aureus* 16/20, *Staph. simulans* 14/29, *Staph. epidermidis* 3/13, *Staphylococcus hyicus* 4/4, and *Staphylococcus devriesei* 2/2.

Group 2. Eleven *Staph. haemolyticus* isolates (91%) and the single *Staph. epidermidis* isolate in group 2 showed resistance to at least 1 of the tested antimicrobials. Resistance to trimethoprim and trimethoprim-sulfonamide were most common, as, in most species including trimethoprim and trimethoprim-sulfonamide–resistant isolates, at least 50% of the isolates were categorized as resistant. Resistance toward cefoxitin was observed in 3 different species: *Staph. cohnii*, *Staph. aureus*, and *Staphylococcus sciuri*; and 18 isolates were multidrug resistant. The multidrug-resistant isolates included the following species: *Staph. cohnii* (n = 5), *Staph. chromogenes* (n = 3), *Staph. equorum* (n = 3), *Staphylococcus arlettae* (n = 2), *Staph. haemolyticus* (n = 2), *Staph. sciuri* (n = 2), and *Staph. aureus* (n = 1). Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. chromogenes* 5/15, *Staph. aureus* 3/12, and *Staph. haemolyticus* 1/11.

Group 3. All *Staph. haemolyticus* isolates (n = 15) and all *Staph. epidermidis* isolates (n = 19) in group 3 showed resistance to at least 1 of the tested antimicrobials. Also, in group 3, resistance to trimethoprim and trimethoprim-sulfonamide were most observed. Resistance toward cefoxitin was observed in 9 different species, including *Staph. aureus*, *Staph. chromogenes*, *Staph. epidermidis*, and *Staph. haemolyticus*. A total of 24 isolates were multidrug resistant, represented by the

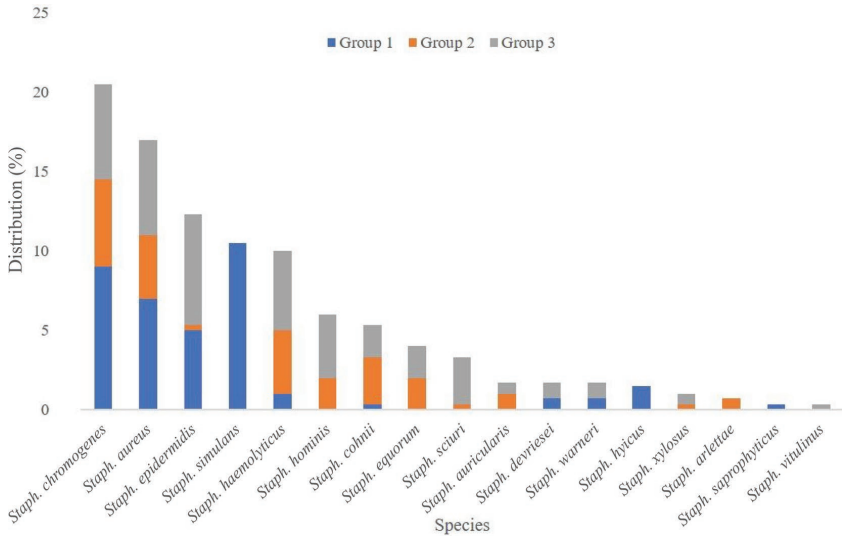


Figure 1. *Staphylococcus* species distribution for the collection of 272 staphylococcal isolates by group, shown as percentage of the total number of isolates. Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia. Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

following species: *Staph. epidermidis* (n = 5), *Staph. aureus* (n = 4), *Staph. haemolyticus* (n = 4), *Staph. sciuri* (n = 4), *Staph. cohnii* (n = 3), *Staphylococcus warneri* (n = 3), and *Staph. hominis* (n = 1). Of these, originating from the same farm, 1 *Staph. epidermidis* isolate had 9 and 2 *Staph. aureus* isolates had 8 observations of resistance toward the panel of tested antimicrobials. This *Staph. epidermidis* isolate was the only isolate phenotypically resistant toward linezolid (Table 3). Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. aureus* 4/16 and *Staph. chromogenes* 3/17.

Figure 2 and Figure 3 show the correlations between the different observed resistances for *Staph. aureus* and the NAS species, respectively. The correlation plots revealed a strong correlation between resistance to trimethoprim and trimethoprim-sulfonamide and between resistance to clindamycin and erythromycin. In addition, we found a stronger correlation between several resistance observations in *Staph. aureus* compared with the NAS species as a group.

Characterization of Isolates with mec Genes

Group 1. No isolates in group 1 were positive for the *mec* genes.

Group 2. The only *mecA*-positive isolate in group 2 was a *Staph. aureus* isolate, which showed resistance to cefoxitin on disk diffusion, as well as resistance to penicillin, ampicillin, and amoxycillin-clavulanic acid, as well as 4 other antimicrobials, as shown in Table 4. No isolates were positive in the *mecC* PCR.

Group 3. In group 3, 4 *Staph. aureus* isolates and 5 NAS isolates were positive in the PCR for the *mecA* gene, but none in the *mecC* PCR. These *mecA*-positive *Staph. aureus* isolates (hereafter referred to as MRSA) originated from the same farm and were thus epidemiologically related. They showed resistance to cefoxitin on disk diffusion; 3 showed resistance to ampicillin; and 2 showed resistance toward amoxicillin-clavulanic acid. These isolates were also multidrug resistant and showed resistance toward 6 or more of the tested antimicrobials. Many of the observed resistances in *Staph. aureus* isolates were traced to these 5 isolates.

The 5 *mecA*-positive NAS belonged to 5 different species: *Staph. epidermidis*, *Staph. haemolyticus*, *Staph. sciuri*, *Staphylococcus vitulinus*, and *Staphylococcus xylosus*. The *Staph. haemolyticus* originated from the same farm as the MRSA, whereas *Staph. epidermidis* came from another farm, and the *Staph. sciuri*, *Staph. vitulinus*, and *Staph. xylosus* from the same third farm. Only the *Staph. haemolyticus* and *Staph. epidermi-*

Table 3. Species distribution and antimicrobial resistance. Species distribution and number of isolates categorized as resistant toward the panel of antimicrobials tested[1]

Species[2]	Total	Ampicillin	Amoxycillin-clavulanic acid	Ciprofloxacin	Clindamycin	Erythromycin	Gentamicin	Linezolid	Penicillin	Trimethoprim-sulfonamide	Trimethoprim	Tetracycline
Group 1												
Staphylococcus aureus	20	1	0	0	0	0	1	0	1	0	1	0
Staphylococcus chromogenes	25	0	0	0	0	2	0	0	4	2	2	0
Staphylococcus epidermidis	13	0	0	0	0	0	0	0	7	5	4	0
Staphylococcus simulans	29	0	0	2	0	1	1	0	1	2	12	0
Staphylococcus hyicus	4	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus haemolyticus	3	0	0	0	0	0	0	0	3	0	0	0
Staphylococcus devriesei	2	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus warneri	2	0	0	0	0	0	1	0	1	0	0	3
Staphylococcus cohnii	1	0	0	0	0	1	0	0	1	0	0	0
Staphylococcus saprophyticus	1	0	0	0	0	0	0	0	1	0	0	0
Group 2												
Staph. aureus	11	1	1	0	0	0	1	0	1	5	8	1
Staph. chromogenes	15	0	0	0	3	3	0	0	5	9	10	3
Staph. haemolyticus	11	0	0	1	2	2	0	0	1	7	9	2
Staph. cohnii	8	0	0	0	0	5	0	0	5	7	8	4
Staphylococcus equorum	5	1	0	0	2	3	0	0	0	3	5	2
Staphylococcus hominis	5	0	0	0	0	0	0	0	1	4	4	2
Staphylococcus auricularis	3	0	0	0	0	0	0	0	0	3	3	0
Staphylococcus arlettae	2	0	0	0	2	2	0	0	2	0	1	0
Staphylococcus sciuri	2	0	0	0	0	0	0	0	2	2	2	2
Staph. epidermidis	1	1	0	0	0	0	0	0	1	0	0	0
Staphylococcus xylosus	1	0	0	0	0	0	0	0	1	0	1	0
Group 3												
Staph. aureus	14	3	2	4	4	4	1	0	4	7	9	0
Staph. epidermidis	19	0	0	2	1	4	3	1	12	18	19	3
Staph. chromogenes	17	0	0	0	0	0	0	0	2	13	13	3
Staph. haemolyticus	15	1	0	0	3	3	0	0	6	15	15	2
Staph. hominis	12	0	0	1	0	1	2	0	0	12	12	4
Staph. sciuri	8	0	0	0	2	0	4	0	4	7	7	4
Staph. cohnii	6	0	0	1	3	3	0	0	5	5	6	1
Staph. equorum	6	0	0	0	0	2	1	0	0	6	6	0
Staph. devriesei	3	0	0	0	0	0	0	0	1	2	3	0
Staph. warneri	3	0	0	0	3	3	0	0	3	0	0	3
Staph. auricularis	2	0	0	0	0	0	0	0	0	2	2	0
Staph. xylosus	2	0	0	0	0	0	0	0	2	1	1	0
Staphylococcus vitulinus	1	0	0	0	0	0	1	0	0	1	1	0

[1]Grey boxes indicate resistant isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

dis isolates showed resistance to cefoxitin. The *Staph. haemolyticus* isolate also showed resistance toward ampicillin, whereas the 4 others were susceptible, and all 5 NAS were phenotypically susceptible toward amoxicillin-clavulanic acid. These isolates also showed resistance toward 4 or fewer of the tested antimicrobials, including trimethoprim-sulfonamide (4/5), penicillin (2/5), gentamicin (1/5), and ampicillin (1/5).

Table 4 shows an overview of the resistance patterns of all *Staph. aureus* isolates.

Virulence Factors

The percentages of *Staph. aureus* isolates in each sample group harboring the different virulence genes are illustrated in Figure 4. All *Staph. aureus* isolates, except one with 2 virulence genes, carried at least 3 of the selected virulence genes. Table 5 shows the presence of virulence genes in a total of 8 NAS isolates.

Group 1. In group 1, 80% of *Staph. aureus* isolates carried at least 4 selected virulence genes. The *cap5* gene was the least frequent gene, followed by the *tsst01* and *fnbpB* genes. One isolate carried 8 of the 9 examined virulence genes, lacking only *cap5*. Only one NAS isolate, a *Staph. chromogenes* isolate, carried any of the virulence genes, namely the *tsst01* gene.

Group 2. A total of 73% of the *Staph. aureus* isolates in group 2 carried 5 or more of the selected virulence genes. The *tsst01* and *fnbpB* genes were the least frequent virulence genes; however, the *tsst01* gene was also carried by the only NAS isolate from group 2 positive for any of the virulence genes tested. This was a *Staph. chromogenes* isolate.

Group 3. All *Staph. aureus* isolates carried 5 or more of the selected virulence genes, of which the *tsst01* and *fnbpB* genes were the least frequent. Three isolates from the same farm carried 7 virulence genes, missing the *tsst01* and *cap8* genes. In this group, 6 NAS iso-

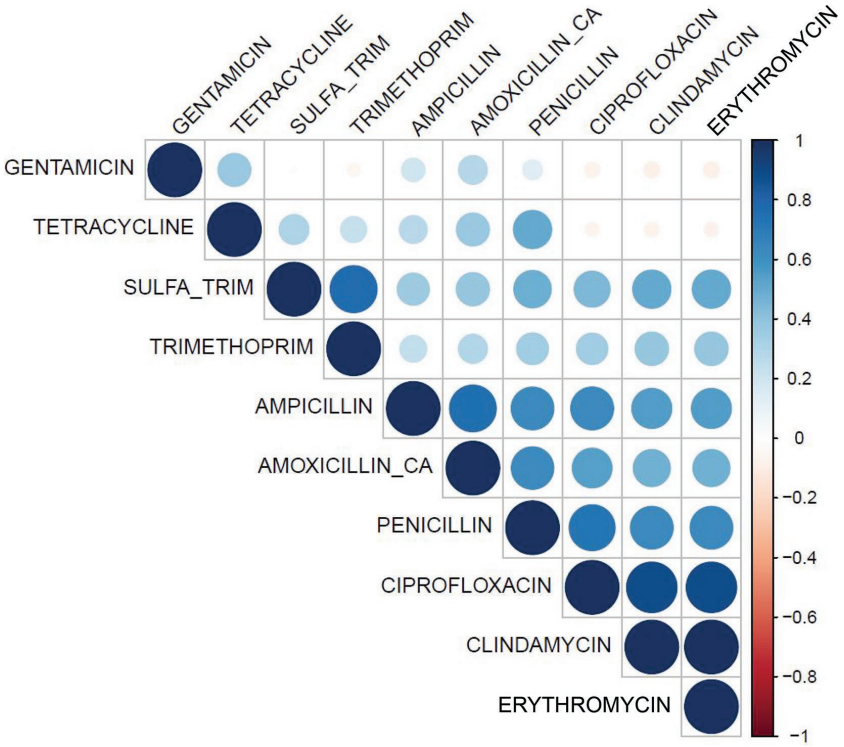


Figure 2. Correlation plot for observed antimicrobial resistances for *Staphylococcus aureus* isolates. Two variables with a phi coefficient >0.7 were considered to be strongly correlated. SULFA_TRIM = sulfonamide and trimethoprim; amoxicillin_ca = amoxicillin and clavulanic acid.

lated carried 1, 5, or 6 of the selected virulence genes, although none with the same profile. This NAS group included 4 *Staph. epidermidis* isolates, 1 *Staph. sciuri* isolate, and 1 *Staph. hominis* isolate.

Figure 5 shows the correlation between the different virulence genes for *Staph. aureus* isolates. We detected little correlation between the virulence genes in *Staph. aureus* isolates. The least frequent virulence genes (*tsst01* and *fnbpB*) as well as the *cap5* gene showed less correlation with other virulence genes. We found a negative correlation between the 2 *cap* genes, *cap5* and *cap8*, in *Staph. aureus* isolates.

Resistance and Virulence

The *Staph. aureus* isolate from group 1 with 8 virulence genes was completely susceptible toward the tested panel of antimicrobials. The 3 isolates from group 3 with 7 virulence genes were multidrug resistant, showing resistance toward 6 or more of the tested antimicrobials, and all 3 harbored the *mecA* gene. The remaining 2 *mecA*-positive *Staph. aureus* isolates carried 6 virulence genes and showed resistance toward 7 of the tested antimicrobials. Out of the 5 NAS that carried 1 virulence gene, one isolate was completely susceptible and the others showed resistance toward 1 to 3 of the tested antimicrobials. However, 1 of these isolates was a *mecA*-positive *Staph. epidermidis*. The other 4 *mecA*-positive NAS did not harbor any of the tested virulence genes. The NAS with 5 virulence genes showed resistance to 4 antimicrobials, and the 2 isolates with 6 virulence genes showed resistance toward 2 or 3 antimicrobials.

DISCUSSION

Methicillin-resistant *Staph. aureus* is a well-recognized and dreaded bacterium threatening both human

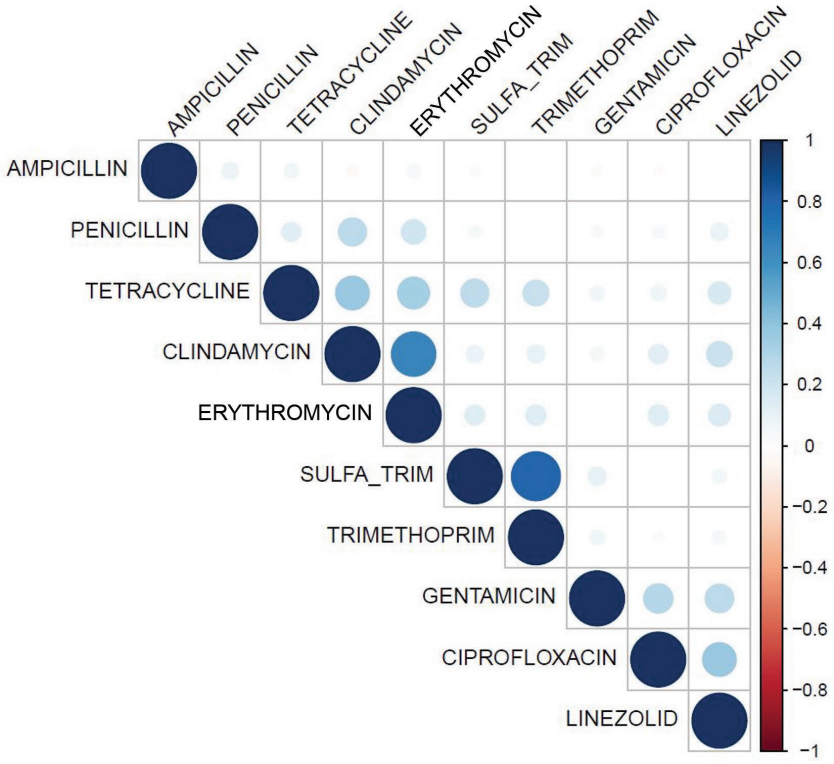


Figure 3. Correlation plot for observed antimicrobial resistances for NAS isolates. Two variables with a phi coefficient greater than 0.7 were considered to be strongly correlated. SULFA_TRIM = sulfonamide and trimethoprim.

Table 4. Number of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staph. aureus* (MSSA) isolates categorized as resistant toward the panel of antimicrobials tested[1]

Species and origin[2]	Total n	Ampicillin	Amoxycillin-clavulanic acid	Ciprofloxacin	Clindamycin	Erythromycin	Gentamicin	Linezolid	Penicillin	Trimethoprim-sulfonamide	Trimethoprim	Tetracycline
MRSA												
Group 1[3]	0	0	0	0	0	0	0	0	0	0	0	0
Group 2	1	1	1	0	0	0	1	0	1	1	1	1
Group 3	4	3	2	4	4	4	0	0	4	4	4	0
MSSA												
Group 1	20	1	0	0	0	0	1	0	1	0	1	0
Group 2	10	0	0	0	0	0	0	0	0	4	7	0
Group 3	10	0	0	0	0	0	1	0	0	3	5	0

[1]Grey boxes indicate resistant isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staph. aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

[3]No isolates of this species.

and animal health, causing potentially serious infections and limited treatment options. In addition, NAS are regarded as a potential reservoir for antimicrobial resistance genes that can be utilized by the more pathogenic *Staph. aureus* (Becker et al., 2014). This study provides findings of MRSA that are both multidrug resistant and harboring several virulence genes, and detection of several resistance traits in NAS isolates belonging to different species. By using 3 distinct collections of staphylococci from bovine milk samples of diverse origins, we were able once again to highlight the presence of antimicrobial resistance characteristics, as well as virulence genes, contributing to an increased knowledge base on this important bacterial group.

Descriptive analyses of antimicrobial resistance characteristics in all 3 sample groups showed that these were more widespread in several NAS species compared with *Staph. aureus*, apart from the MRSA isolates. This distribution corresponds with previous findings in Norwegian and Dutch surveillance systems for use of antimicrobial agents and occurrence of antimicrobial resistance (Mevius et al., 2007, 2008; NORM/NORM-VET, 2015). Non-*aureus* staphylococci are believed to represent an important reservoir for antimicrobial resistance (Becker et al., 2014), and NAS isolates from group 2 and group 3 in this study were more frequently multidrug resistant compared with their group-corresponding *Staph. aureus* isolates. Antimicrobial resistance was frequently observed in *Staph. epidermidis* and *Staph. haemolyticus* regardless of sample group, the "worst case" being the one *Staph. epidermidis* isolate from group 3 that was resistant to 9 of the 11 tested antimicrobials and the only isolate phenotypically resistant to linezolid. However, this isolate was not *mecA* positive. The finding of multidrug-resistant *Staph. epidermidis* has been shown previously (Nobrega et al., 2018). Although the milk food chain is not regarded as a major transfer route for antimicrobial-resistant bacteria because the pasteurization process will kill vegetative bacteria, the fact that *Staph. epidermidis* and *Staph. haemolyticus* are common causes of nosocomial infections in humans (Spanu et al., 2003; Huang et al., 2005; Shin et al., 2011) suggests that resistant NAS from dairy cattle could potentially be a public health hazard.

The high prevalence of resistance toward trimethoprim and trimethoprim-sulfonamide in groups 2 and 3 was surprising. This contrasts with findings from other European studies on staphylococci, where resistance toward penicillin is most prevalent (Botrel et al., 2010; Persson Waller et al., 2011; Taponen et al., 2016). A survey conducted among 3,000 practitioners in 25 European countries reported that β-lactams, mainly penicillin, are the drugs of choice when treating bovine

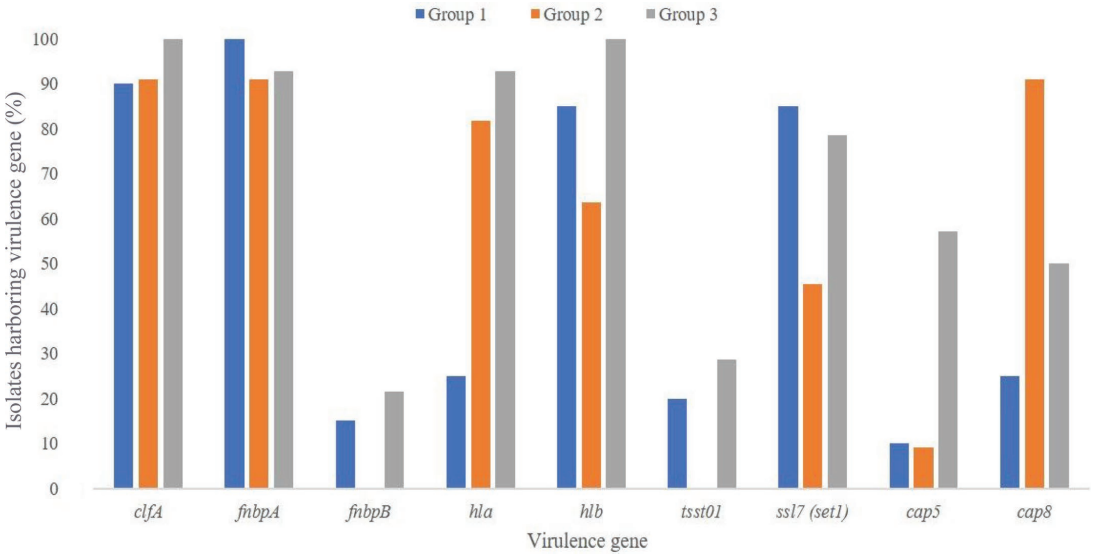


Figure 4. Percentage of *Staphylococcus aureus* isolates harboring the different virulence genes. Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staph. aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite SCC >300,000 cells/mL from 3 different herds.

mastitis (De Briyne et al., 2014). In the European Surveillance of Veterinary Antimicrobial Consumption report of 2017 on sales of veterinary antimicrobial agents for food-producing animals, measured in milligrams per population-correction unit (mg/PCU), penicillin is the most sold drug in Norway and Belgium, followed by tetracyclines and sulfonamides in Belgium, and sulfonamides, amphenicols, and aminoglycosides in Norway

Table 5. Presence of virulence genes in single non-*aureus* staphylococcal isolates[1]

Species, origin[2]	*cap5*	*cap8*	*clfA*	*fnbpA*	*fnbpB*	*hla*	*hlb*	*tsst01*	*ssl7* (*set1*)
Staphylococcus chromogenes Group 1	−	−	−	−	−	−	−	+	−
Staph. chromogenes Group 2	−	−	−	−	−	−	−	+	−
Staphylococcus sciuri Group 3	+	−	−	−	−	−	−	−	−
Staphylococcus epidermidis Group 3	−	−	−	−	−	+	−	−	−
Staph. epidermidis Group 3	+	−	−	−	−	−	−	−	−
Staph. epidermidis Group 3	−	+	+	+	−	+	+	−	−
Staph. epidermidis Group 3	−	+	+	+	−	−	+	+	+
Staphylococcus hominis Group 3	−	+	+	+	−	+	+	+	−

[1]Grey boxes indicate presence of virulence genes in individual isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

(European Medicines Agency, 2019), although the use of amphenicols in Norway is mostly related to farmed fish (Lillehaug et al., 2018). The survey from De Briyne et al. (2014) indicates that most practitioners report prescribing sulfonamides for pigs, not cattle. However, there is not always a simple link between the use of one antimicrobial and subsequent development of resistance toward the same antimicrobial. Studies of *E. coli* from horses and calves have shown that treatment with penicillin leads to increased phenotypic resistance to multiple unrelated antimicrobials (Grønvold et al., 2010; Grønvold et al., 2011). If similar, and still cryptic, relations between use and resistance also holds true for staphylococci, it may be one explanation of the high prevalence of observed trimethoprim and trimethoprim-sulfonamide resistance. Resistance toward penicillin was most common in group 1, except in the *Staph. simulans* isolates, and second most common in groups 2 and 3, more in accordance with reported prescription patterns. Some of the observed differences in antimicrobial resistance between group 1 and groups 2 and 3 might be related to differences between the countries, as Norway generally has a low prevalence of antimicrobial resistance in dairy herds (NORM/NORM-VET, 2017).

The strong correlation between erythromycin and clindamycin resistance observed in this study is consistent with what has been shown in other studies (Lüthje and Schwarz, 2006; Li et al., 2015). This is most likely due to *erm* genes that generally confer resistance toward both macrolides and lincosamides, as well as streptogramin B. These genes are commonly found in staphylococci and are often located on mobile genetic elements, which could allow for horizontal spread of the genes (Feßler et al., 2018). It is interesting to note that stronger correlations seem to exist between the observed resistances in the *Staph. aureus* isolates compared with the NAS isolates. This could possibly be related to the plasticity of the *Staph. aureus* pan genome, with mobile elements readily exchanged between *Staph. aureus* isolates, and resistance genes possibly

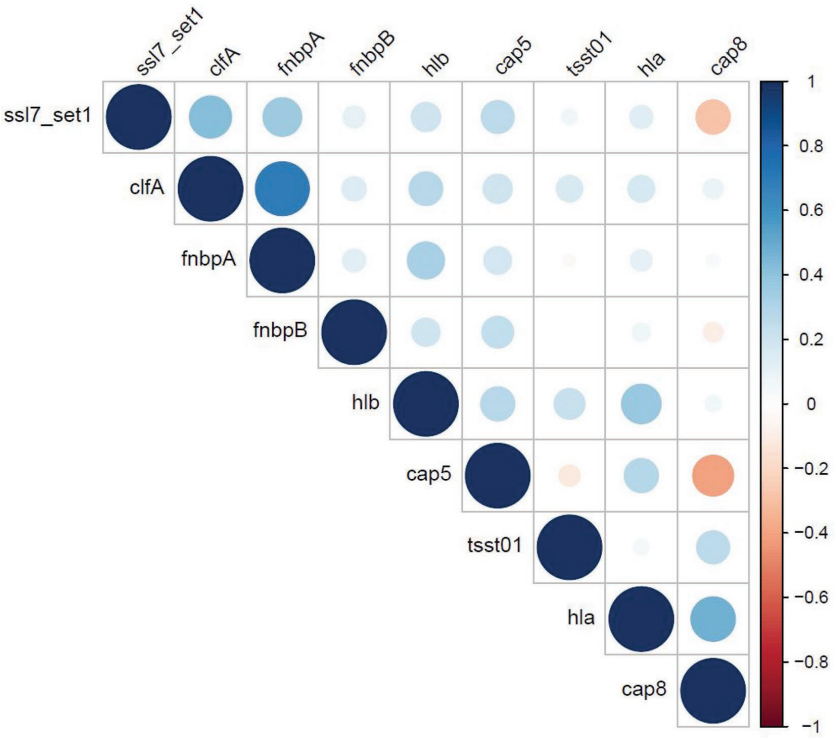


Figure 5. Correlation plot of virulence genes for *Staphylococcus aureus* isolates. Two variables with a phi coefficient >0.7 were considered to be strongly correlated.

co-localized on these mobile elements (Holden et al., 2004; Lindsay and Holden, 2004). Because this study mostly tested phenotypic resistance, it is also possible that some isolates carry resistance mechanisms conferring resistance to multiple antimicrobial classes (Wendlandt et al., 2015). Although one may assume that these genes and elements spread between staphylococcal species, it is possible that the more pathogenic *Staph. aureus* is more often exposed to antimicrobial treatments and therefore more frequently exchanges mobile elements containing resistance genes. However, because NAS are often found as commensals, it could be expected that these staphylococci are constantly exposed to systemically administered antimicrobial agents; this has also been proposed in another study (Stevens et al., 2018). This assumption may contribute to explain the development of NAS as a reservoir for antimicrobial resistance.

A previous study of Belgian farms suffering from *Staph. aureus* mastitis indicated that about 10% of these *Staph. aureus* isolates were MRSA (Vanderhaeghen et al., 2010). As documented, MRSA strains display resistance to a variety of antimicrobial agents, in addition to their resistance to β-lactams (Vanderhaeghen et al., 2010; Bardiau et al., 2013). Our results are consistent with these observations, as the 5 MRSA showed resistance to 6 to 8 of the tested antimicrobials. The high prevalence of antimicrobial resistance in MRSA is a serious problem, limiting treatment options and threatening both animal and human health.

The 5 MRSA isolates were concordantly resistant to cefoxitin on disk diffusion, yet only 2 of the *mecA*-positive NAS showed this resistance. The possibility exists that these NAS isolates may carry a variant of the *mecA* gene that does not confer resistance to cefoxitin. Studies regarding the evolution of *mecA* and the mobile genetic cassette that carries the gene in staphylococci have found that most *Staph. sciuri* harbor a gene, *mecA1*, with 80% homology to *mecA* in *Staph. aureus*. However, this gene generally does not confer methicillin resistance (Couto et al., 1996; Wu et al., 1996). It is also possible that the *mecA* gene is truncated or not expressed in these isolates. In addition, 15 NAS isolates were phenotypically resistant to cefoxitin without a positive PCR for the *mecA* gene. The explanation of why some of the NAS isolates did not show phenotypic cefoxitin resistance despite harboring a *mecA* gene and others showed resistance without being *mecA*-positive on PCR is not known at this stage and warrants further genetic studies. For example, some reports have found a plasmid-encoded *mecB* gene in *Staph. aureus* isolates, which, if present in the cefoxitin-resistant NAS, could explain the negative PCR for *mecA* and *mecC* (Becker et al., 2018).

We found a high prevalence of several virulence genes in *Staph. aureus* in all groups, especially *clfA*, *fnbpA*, and *hlb*, as well as a low occurrence of *fnbpB*. These patterns are in accordance with other studies of both bovine and human *Staph. aureus* isolates (Booth et al., 2001; Salasia et al., 2004). We observed a higher frequency of *hlb* compared with *hla* in both groups of staphylococci collected from cows with mastitis or high cow SCC (groups 1 and 3). This contrasts with findings from clinical human isolates, were *hla* appears more common (Booth et al., 2001). However, Aarestrup et al. (1999) found the *hlb* gene to be significantly more prevalent in bovine isolates compared with human isolates. This indicates that *hlb* might play a more important role in the pathogenicity of *Staph. aureus* in bovine mastitis, which is also proposed in another study (Resch et al., 2013). We detected a strong correlation between several different virulence genes, especially *ssl7* (*set1*), *hlb*, *clfA*, and *fnbpA*, further indicating that these genes are important determinants in the virulence of *Staph. aureus* associated with the bovine udder. Adherence to extracellular matrix proteins is believed to be crucial for the ability of *Staph. aureus* to colonize and invade tissue (Cremonesi et al., 2013). Fibronectin-binding proteins play a significant role in bacterial adhesion and invasion of the bovine mammary gland (Lammers et al., 1999), and the *clfA* and *clfB* genes are also associated with the initial adherence of *Staph. aureus* to the teat canal (da Costa et al., 2014). This makes *Staph. aureus* harboring *clfA* and *fnbpB* especially fit for invasion (Cremonesi et al., 2013). The *ssl7* (*set1*) gene represents a group of genes encoding staphylococcal superantigen-like proteins that shares homology with other superantigens (Langley et al., 2005). The gene encodes a protein that contributes to bacterial immune evasion, such as inhibition of phagocytosis and cytokine and chemokine secretion (Wines et al., 2011). The role of superantigens and superantigen-like proteins in the pathogenesis of bovine mastitis is not fully known. However, indications exist that these proteins play a role in bovine mastitis, inducing tissue damage and inflammation, as well as immunosuppression and immune evasion (Wilson et al., 2018). This study further supports the virulence potential of *Staph. aureus*, with many of the isolates carrying several virulence genes. It is worth noting that the 5 *mecA*-positive *Staph. aureus* isolates harbored 7 or 8 of the screened virulence genes, in addition to showing phenotypic resistance toward 6 or more of the tested antimicrobials. These isolates pose serious challenges for the management of individual udder infections, as the bacteria have a high pathogenic potential and are difficult to treat.

The screened virulence genes were derived from studies on *Staph. aureus*, potentially leading to a lower de-

tection of these genes in NAS species. Compared with *Staph. aureus*, less is known about the pathogenicity of NAS, but they possess fewer virulence properties than *Staph. aureus* (Becker et al., 2014). In more recent studies, virulence genes in NAS have been identified by whole-genome sequencing of different collections of isolates (Åvall-Jääskeläinen et al., 2018; Naushad et al., 2019), but no conclusive findings about gene content and virulence have been made.

The isolates in this study were collected from dairy farms in Norway and 2 regions (Flanders and Wallonia) in Belgium, but, as the sampling regimens were different for the 3 geographical areas, the results are not comparable between regions. The main reasons for the variability in sampling strategies were the different structures of the dairy farm industry in Norway versus Belgium, and the accessibility to farms. These differences in sampling strategies might have affected the distribution of NAS species in the different groups. In group 1, NAS species were isolated from samples primarily sent to the laboratory due to high SCC or clinical mastitis, whereas in groups 2 and 3 NAS were collected from samples from all quarters of each animal. This could explain why *Staph. simulans* was more frequently isolated in group 1 and *Staph. cohnii* more prevalent in groups 2 and 3, as *Staph. simulans* has been shown to be associated with a higher SCC compared with several other NAS species and *Staph. cohnii* more frequently associated with low SCC (Supré et al., 2011; Condas et al., 2017). There is also a possibility that isolates collected from different cows in groups 2 and 3 are part of the same strain, so-called copy strains, as *Staph. aureus* is considered a contagious pathogen (Kirkeby et al., 2019) transmitted from cow to cow.

CONCLUSIONS

By using 3 distinct collections of staphylococci from bovine milk samples of diverse origins, we were able once again to highlight the presence of antimicrobial resistance characteristics, as well as virulence genes, contributing to an increased knowledge base of this important bacterial group. Antimicrobial resistance characteristics in all 3 sample groups were more widespread in several NAS species compared with *Staph. aureus*, apart from the MRSA isolates. In all groups, antimicrobial resistance was common in *Staph. epidermidis* and *Staph. haemolyticus*. Only a few NAS isolates carried any of the virulence genes typically displayed by *Staph. aureus*. Even though the results for the MRSA in this study are concerning with regard to multidrug resistance, most of the *Staph. aureus* collected did not carry the *mecA* gene and showed relatively little resistance, giving hope that it is still possible to slow the development and spread of MRSA among dairy cattle.

ACKNOWLEDGMENTS

This project was part of the ERA-Net Animal Health and Welfare (ANIHWA), MRSA_bacteriophages project. The project was funded by the Research Council of Norway, the Research Foundation – Flanders (FWO), and Service Public Fédéral – Santé publique, Sécurité de la Chaîne alimentaire et Environnement (Wallonia). Salary was provided by each home institution. We thank Ola B. Brynildsrud (Norwegian Institute of Public Health, Norway) for assistance with correlation plots. We are very grateful for the laboratory help from Olga Anna Osinska (Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, currently Norwegian Institute of Public Health, Norway). We thank Olav Østerås (Dairy Advisory Services, TINE SA, Norway) for valuable input to the manuscript. We thank Anne-Sophie Rao (Department of Farm Animal Clinics, Faculty of Veterinary Medicine, University of Liège, Belgium) and the MammiScan project (SPW-DGARNE) for the milk sampling in Wallonia. The authors have not stated any conflicts of interest.

REFERENCES

Aarestrup, F. M., H. D. Larsen, N. H. Eriksen, C. S. Elsberg, and N. E. Jensen. 1999. Frequency of α- and β-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. APMIS 107:425–430. https://doi.org/10.1111/j.1699-0463.1999.tb01576.x.

Åvall-Jääskeläinen, S., S. Taponen, R. Kant, L. Paulin, J. Blom, A. Palva, and J. Koort. 2018. Comparative genome analysis of 24 bovine-associated *Staphylococcus* isolates with special focus on the putative virulence genes. PeerJ 6:e4560. https://doi.org/10.7717/peerj.4560.

Bardiau, M., K. Yamazaki, J. N. Duprez, B. Taminiau, J. G. Mainil, and I. Ote. 2013. Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. Lett. Appl. Microbiol. 57:181–186. https://doi.org/10.1111/lam.12099.

Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493–496.

Becker, K., C. Heilmann, and G. Peters. 2014. Coagulase-negative staphylococci. Clin. Microbiol. Rev. 27:870–926. https://doi.org/10.1128/CMR.00109-13.

Becker, K., S. van Alen, E. A. Idelevich, N. Schleimer, J. Seggewiß, A. Mellmann, U. Kaspar, and G. Peters. 2018. Plasmid-encoded transferable mecB-mediated methicillin resistance in *Staphylococcus aureus*. Emerg. Infect. Dis. 24:242–248. https://doi.org/10.3201/eid2402.171074.

Booth, M. C., L. M. Pence, P. Mahasreshti, M. C. Callegan, and M. S. Gilmore. 2001. Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. Infect. Immun. 69:345–352. https://doi.org/10.1128/IAI.69.1.345-352.2001.

Botrel, M. A., M. Haenni, E. Morignat, P. Sulpice, J. Y. Madec, and D. Calavas. 2010. Distribution and antimicrobial resistance of

clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Foodborne Pathog. Dis. 7:479–487. https://doi.org/10.1089/fpd.2009.0425.

Cameron, M., H. W. Barkema, J. De Buck, S. De Vliegher, M. Chaffer, J. Lewis, and G. P. Keefe. 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. J. Dairy Sci. 100:2137–2147. https://doi.org/10.3168/jds.2016-12020.

Cameron, M., J. Perry, J. R. Middleton, M. Chaffer, J. Lewis, and G. P. Keefe. 2018. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. J. Dairy Sci. 101:590–595. https://doi.org/10.3168/jds.2017-13226.

Condas, L. A. Z., J. De Buck, D. B. Nobrega, D. A. Carson, J. P. Roy, G. P. Keefe, T. J. DeVries, J. R. Middleton, S. Dufour, and H. W. Barkema. 2017. Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. J. Dairy Sci. 100:5613–5627. https://doi.org/10.3168/jds.2016-12479.

Couto, I., H. de Lencastre, E. Severina, W. Kloos, J. A. Webster, R. J. Hubner, I. S. Sanches, and A. Tomasz. 1996. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. Microb. Drug Resist. 2:377–391. https://doi.org/10.1089/mdr.1996.2.377.

Cremonesi, P., T. Zottola, C. Locatelli, C. Pollera, B. Castiglioni, L. Scaccabarozzi, and P. Moroni. 2013. Identification of virulence factors in 16S–23S rRNA intergenic spacer genotyped *Staphylococcus aureus* isolated from water buffaloes and small ruminants. J. Dairy Sci. 96:7666–7674. https://doi.org/10.3168/jds.2013-6917.

da Costa, L. B., P. J. Rajala-Schultz, A. Hoet, K. S. Seo, K. Fogt, and B. S. Moon. 2014. Genetic relatedness and virulence factors of bovine *Staphylococcus aureus* isolated from teat skin and milk. J. Dairy Sci. 97:6907–6916. https://doi.org/10.3168/jds.2014-7972.

Dalen, G., A. Rachah, H. Nørstebø, Y. H. Schukken, and O. Reksen. 2019. The detection of intramammary infections using online somatic cell counts. J. Dairy Sci. 102:5419–5429. https://doi.org/10.3168/jds.2018-15295.

De Briyne, N., J. Atkinson, S. P. Borriello, and L. Pokludová. 2014. Antibiotics used most commonly to treat animals in Europe. Vet. Rec. 175:325. https://doi.org/10.1136/vr.102462.

De Visscher, A., F. Haesebrouck, S. Piepers, W. Vanderhaeghen, K. Supré, F. Leroy, E. Van Coillie, and S. De Vliegher. 2013. Assessment of the suitability of mannitol salt agar for growing bovine-associated coagulase-negative staphylococci and its use under field conditions. Res. Vet. Sci. 95:347–351. https://doi.org/10.1016/j.rvsc.2013.05.015.

De Visscher, A., S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016. Intramammary infection with coagulase-negative staphylococci at parturition: Species-specific prevalence, risk factors, and effect on udder health. J. Dairy Sci. 99:6457–6469. https://doi.org/10.3168/jds.2015-10458.

De Vliegher, S., L. K. Fox, S. Piepers, S. McDougall, and H. W. Barkema. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. J. Dairy Sci. 95:1025–1040. https://doi.org/10.3168/jds.2010-4074.

El Garch, F., M. Youala, S. Simjee, H. Moyaert, R. Klee, B. Truszkowska, M. Rose, D. Hocquet, B. Valot, I. Morrissey, and A. de Jong. 2020. Antimicrobial susceptibility of nine udder pathogens recovered from bovine clinical mastitis milk in Europe 2015–2016: VetPath results. Vet. Microbiol. 245:108644. https://doi.org/10.1016/j.vetmic.2020.108644.

European Medicines Agency. 2019. Sales of veterinary antimicrobial agents in 31 European countries in 2017. European Surveillance of Veterinary Antimicrobial Consumption. Accessed Apr. 15, 2021. https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2018-trends-2010-2018-tenth-esvac-report_en.pdf.

Feßler, A. T., C. Billerbeck, K. Kadlec, and S. Schwarz. 2010. Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis. J. Antimicrob. Chemother. 65:1576–1582. https://doi.org/10.1093/jac/dkq172.

Feßler, A. T., Y. Wang, C. Wu, and S. Schwarz. 2018. Mobile lincosamide resistance genes in staphylococci. Plasmid 99:22–31. https://doi.org/10.1016/j.plasmid.2018.06.002.

Gao, J., H. W. Barkema, L. Zhang, G. Liu, Z. Deng, L. Cai, R. Shan, S. Zhang, J. Zou, J. P. Kastelic, and B. Han. 2017. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. J. Dairy Sci. 100:4797–4806. https://doi.org/10.3168/jds.2016-12334.

Gindonis, V., S. Taponen, A. L. Myllyniemi, S. Pyörälä, S. Nykäsenoja, S. Salmenlinna, L. Lindholm, and M. Rantala. 2013. Occurrence and characterization of methicillin-resistant staphylococci from bovine mastitis milk samples in Finland. Acta Vet. Scand. 55:61. https://doi.org/10.1186/1751-0147-55-61.

Grønvold, A. M., T. M. L'Abée-Lund, E. Strand, H. Sørum, A. C. Yannarell, and R. I. Mackie. 2010. Fecal microbiota of horses in the clinical setting: Potential effects of penicillin and general anesthesia. Vet. Microbiol. 145:366–372. https://doi.org/10.1016/j.vetmic.2010.03.023.

Grønvold, A. M., Y. Mao, T. M. L'Abée-Lund, H. Sørum, T. Sivertsen, A. C. Yannarell, and R. I. Mackie. 2011. Fecal microbiota of calves in the clinical setting: Effect of penicillin treatment. Vet. Microbiol. 153:354–360. https://doi.org/10.1016/j.vetmic.2011.05.040.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29:18–31. https://doi.org/10.1080/01652176.2007.9695224.

Hanssen, A. M., and J. U. Ericson Sollid. 2006. SCCmec in staphylococci: Genes on the move. FEMS Immunol. Med. Microbiol. 46:8–20. https://doi.org/10.1111/j.1574-695X.2005.00009.x.

Heikkilä, A. M., E. Liski, S. Pyörälä, and S. Taponen. 2018. Pathogen-specific production losses in bovine mastitis. J. Dairy Sci. 101:9493–9504. https://doi.org/10.3168/jds.2018-14824.

Holden, M. T., E. J. Feil, J. A. Lindsay, S. J. Peacock, N. P. Day, M. C. Enright, T. J. Foster, C. E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S. D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K. D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B. G. Barrell, B. G. Spratt, and J. Parkhill. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: Evidence for the rapid evolution of virulence and drug resistance. Proc. Natl. Acad. Sci. USA 101:9786–9791. https://doi.org/10.1073/pnas.0402521101.

Huang, C. R., C. H. Lu, J. J. Wu, H. W. Chang, C. C. Chien, C. B. Lei, and W. N. Chang. 2005. Coagulase-negative staphylococcal meningitis in adults: Clinical characteristics and therapeutic outcomes. Infection 33:56–60. https://doi.org/10.1007/s15010-005-4083-7.

Kadlec, K., A. T. Fessler, T. Hauschild, and S. Schwarz. 2012. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant Staphylococcus aureus. Clin. Microbiol. Infect. 18:745–755. https://doi.org/10.1111/j.1469-0691.2012.03842.x.

Kirkeby, C., L. Zervens, N. Toft, D. Schwarz, M. Farre, S. Hechinger, and T. Halasa. 2019. Transmission dynamics of *Staphylococcus aureus* within two Danish dairy cattle herds. J. Dairy Sci. 102:1428–1442. https://doi.org/10.3168/jds.2018-15106.

Lammers, A., P. J. Nuijten, and H. E. Smith. 1999. The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion to and invasion of bovine mammary gland cells. FEMS Microbiol. Lett. 180:103–109. https://doi.org/10.1111/j.1574-6968.1999.tb08783.x.

Langley, R., B. Wines, N. Willoughby, I. Basu, T. Proft, and J. D. Fraser. 2005. The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. J. Immunol. 174:2926–2933. https://doi.org/10.4049/jimmunol.174.5.2926.

Lee, J. H. 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential trans-

mission to humans. Appl. Environ. Microbiol. 69:6489–6494. https://doi.org/10.1128/AEM.69.11.6489-6494.2003.

Levy, S. B., and B. Marshall. 2004. Antibacterial resistance worldwide: Causes, challenges and responses. Nat. Med. 10(Suppl. 12):S122–S129. https://doi.org/10.1038/nm1145.

Li, L., W. Feng, Z. Zhang, H. Xue, and X. Zhao. 2015. Macrolide-lincosamide-streptogramin resistance phenotypes and genotypes of coagulase-positive *Staphylococcus aureus* and coagulase-negative staphylococcal isolates from bovine mastitis. BMC Vet. Res. 11:168. https://doi.org/10.1186/s12917-015-0492-8.

Lillehaug, A., C. Børnes, and K. Grave. 2018. A pharmaco-epidemiological study of antibacterial treatments and bacterial diseases in Norwegian aquaculture from 2011 to 2016. Dis. Aquat. Organ. 128:117–125. https://doi.org/10.3354/dao03219.

Lindsay, J. A., and M. T. Holden. 2004. *Staphylococcus aureus*: Superbug, super genome? Trends Microbiol. 12:378–385. https://doi.org/10.1016/j.tim.2004.06.004.

Lüthje, P., and S. Schwarz. 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. J. Antimicrob. Chemother. 57:966–969. https://doi.org/10.1093/jac/dkl061.

Mevius, D., M. G. J. Koene, L. F. Puister, R. H. M. Bergevoet, H. B. van der Veen, and I. van Geijlswijk. MARAN 2008: Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2008. Accessed Apr. 8, 2021. https://www.wur.nl/upload_mm/7/6/a/5becb8c3-70be-4930-ae2f-397760c2b440_MARAN2008.pdf.

Mevius, D., B. Wit, W. Van Pelt, L. F. Puister-Jansen, N. Bondt, R. H. M. Bergevoet, and I. M. Van Geijlswijk. MARAN 2007: Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2006/2007. Accessed Apr. 8, 2021. https://www.wur.nl/upload_mm/a/7/3/049cc3d3-86d8-4352-a889-539c97849628_MARAN2006-2007.pdf.

Mikalsen, V., O. Østerås, and T. Roalkvam. 2019. Statistics collection: Statistikksamling fra Ku- og Geitekontrollen 2019. Husdyrkontrollen, TINE Rådgivning, Norway. Accessed Apr. 15, 2021. https://www.geno.no/contentassets/f2a65a5153e04bf299653d9fde0c1151/statistikksamling-2019-ku-og-geitekontrollen.pdf.

Naushad, S., S. A. Naqvi, D. Nobrega, C. Luby, J. P. Kastelic, H. W. Barkema, and J. De Buck. 2019. Comprehensive virulence gene profiling of bovine non-*aureus* staphylococci based on whole-genome sequencing data. mSystems 4:e00098-18. https://doi.org/10.1128/mSystems.00098-18.

Nobrega, D. B., J. De Buck, and H. W. Barkema. 2018. Antimicrobial resistance in non-*aureus* staphylococci isolated from milk is associated with systemic but not intramammary administration of antimicrobials in dairy cattle. J. Dairy Sci. 101:7425–7436. https://doi.org/10.3168/jds.2018-14540.

Nobrega, D. B., J. De Buck, S. A. Naqvi, G. Liu, S. Naushad, V. Saini, and H. W. Barkema. 2017. Comparison of treatment records and inventory of empty drug containers to quantify antimicrobial usage in dairy herds. J. Dairy Sci. 100:9736–9745. https://doi.org/10.3168/jds.2017-13116.

NORM/NORM-VET. 2015. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø/Oslo 2016. Accessed Apr. 8, 2021. https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report.

NORM/NORM-VET. 2017. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway Tromsø/Oslo 2018. Accessed Apr. 8, 2021. https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report.

Olde Riekerink, R. G., H. W. Barkema, D. F. Kelton, and D. T. Scholl. 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J. Dairy Sci. 91:1366–1377. https://doi.org/10.3168/jds.2007-0757.

Oliver, S. P., and S. E. Murinda. 2012. Antimicrobial resistance of mastitis pathogens. Vet. Clin. North Am. Food Anim. Pract. 28:165–185. https://doi.org/10.1016/j.cvfa.2012.03.005.

Østerås, O., L. Sølverød, and O. Reksen. 2006. Milk culture results in a large Norwegian survey—Effects of season, parity, days in milk, resistance, and clustering. J. Dairy Sci. 89:1010–1023. https://doi.org/10.3168/jds.S0022-0302(06)72167-1.

Ote, I., B. Taminiau, J. N. Duprez, I. Dizier, and J. G. Mainil. 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153:285–292. https://doi.org/10.1016/j.vetmic.2011.05.042.

Persson Waller, K., A. Aspán, A. Nyman, Y. Persson, and U. Grönlund Andersson. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Vet. Microbiol. 152:112–116. https://doi.org/10.1016/j.vetmic.2011.04.006.

Piepers, S., L. De Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. De Vliegher. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J. Dairy Res. 74:478–483. https://doi.org/10.1017/S0022029907002841.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Resch, G., P. François, D. Morisset, M. Stojanov, E. J. Bonetti, J. Schrenzel, O. Sakwinska, and P. Moreillon. 2013. Human-to-bovine jump of *Staphylococcus aureus* CC8 is associated with the loss of a β-hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. PLoS One 8:e58187. https://doi.org/10.1371/journal.pone.0058187.

Rollin, E., K. C. Dhuyvetter, and M. W. Overton. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Prev. Vet. Med. 122:257–264. https://doi.org/10.1016/j.prevetmed.2015.11.006.

Saini, V., J. T. McClure, D. Léger, S. Dufour, A. G. Sheldon, D. T. Scholl, and H. W. Barkema. 2012. Antimicrobial use on Canadian dairy farms. J. Dairy Sci. 95:1209–1221. https://doi.org/10.3168/jds.2011-4527.

Salasia, S. I., Z. Khusnan, C. Lammler, and M. Zschock. 2004. Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. J. Vet. Sci. 5:103–109. https://doi.org/10.4142/jvs.2004.5.2.103.

Schoenfelder, S. M., Y. Dong, A. T. Feßler, S. Schwarz, C. Schoen, R. Köck, and W. Ziebuhr. 2017. Antibiotic resistance profiles of coagulase-negative staphylococci in livestock environments. Vet. Microbiol. 200:79–87. https://doi.org/10.1016/j.vetmic.2016.04.019.

Shin, J. H., S. H. Kim, H. S. Jeong, S. H. Oh, H. R. Kim, J. N. Lee, Y. C. Yoon, Y. W. Kim, and Y. H. Kim. 2011. Identification of coagulase-negative staphylococci isolated from continuous ambulatory peritoneal dialysis fluid using 16S ribosomal RNA, tuf, and SodA gene sequencing. Perit. Dial. Int. 31:340–346. https://doi.org/10.3747/pdi.2010.00073.

Spanu, T., M. Sanguinetti, D. Ciccaglione, T. D'Inzeo, L. Romano, F. Leone, and G. Fadda. 2003. Use of the VITEK 2 system for rapid identification of clinical isolates of staphylococci from bloodstream infections. J. Clin. Microbiol. 41:4259–4263. https://doi.org/10.1128/JCM.41.9.4259-4263.2003.

Stegger, M., P. S. Andersen, A. Kearns, B. Pichon, M. A. Holmes, G. Edwards, F. Laurent, C. Teale, R. Skov, and A. R. Larsen. 2012. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mecALGA251*. Clin. Microbiol. Infect. 18:395–400. https://doi.org/10.1111/j.1469-0691.2011.03715.x.

Stevens, M., S. Piepers, K. Supré, and S. De Vliegher. 2018. Antimicrobial consumption on dairy herds and its association with antimicrobial inhibition zone diameters of non-aureus staphylococci and *Staphylococcus aureus* isolated from subclinical mastitis. J. Dairy Sci. 101:3311–3322. https://doi.org/10.3168/jds.2017-13365.

Stevens, M., S. Piepers, K. Supré, J. Dewulf, and S. De Vliegher. 2016. Quantification of antimicrobial consumption in adult cattle on dairy herds in Flanders, Belgium, and associations with udder health, milk quality, and production performance. J. Dairy Sci. 99:2118–2130. https://doi.org/10.3168/jds.2015-10199.

Supré, K., S. De Vliegher, O. C. Sampimon, R. N. Zadoks, M. Vaneechoutte, M. Baele, E. De Graef, S. Piepers, and F. Hae-

sebrouck. 2009. Technical note: Use of transfer RNA-intergenic spacer PCR combined with capillary electrophoresis to identify coagulase-negative *Staphylococcus* species originating from bovine milk and teat apices. J. Dairy Sci. 92:3204–3210. https://doi.org/10.3168/jds.2008-1923.

Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vaneechoutte, S. Piepers, and S. De Vliegher. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. J. Dairy Sci. 94:2329–2340. https://doi.org/10.3168/jds.2010-3741.

Tacconelli, E. 2009. Antimicrobial use: Risk driver of multidrug resistant microorganisms in healthcare settings. Curr. Opin. Infect. Dis. 22:352–358. https://doi.org/10.1097/QCO.0b013e32832d52e0.

Taponen, S., S. Nykäsenoja, T. Pohjanvirta, A. Pitkälä, and S. Pyörälä. 2016. Species distribution and in vitro antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitic milk. Acta Vet. Scand. 58:12. https://doi.org/10.1186/s13028-016-0193-8.

Unal, S., J. Hoskins, J. E. Flokowitsch, C. Y. Wu, D. A. Preston, and P. L. Skatrud. 1992. Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. J. Clin. Microbiol. 30:1685–1691.

Valckenier, D., S. Piepers, A. De Visscher, R. M. Bruckmaier, and S. De Vliegher. 2019. Effect of intramammary infection with non-*aureus* staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. J. Dairy Sci. 102:6442–6453. https://doi.org/10.3168/jds.2018-15913.

Vanderhaeghen, W., T. Cerpentier, C. Adriaensen, J. Vicca, K. Hermans, and P. Butaye. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbiol. 144:166–171. https://doi.org/10.1016/j.vetmic.2009.12.044.

Verbeke, J., S. Piepers, K. Supré, and S. De Vliegher. 2014. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. J. Dairy Sci. 97:6926–6934. https://doi.org/10.3168/jds.2014-8173.

Waage, S., T. Mørk, A. Røros, D. Aasland, A. Hunshamar, and S. A. Odegaard. 1999. Bacteria associated with clinical mastitis in dairy heifers. J. Dairy Sci. 82:712–719. https://doi.org/10.3168/jds.S0022-0302(99)75288-4.

Wang, D., L. Zhang, X. Zhou, Y. He, C. Yong, M. Shen, O. Szenci, and B. Han. 2016. Antimicrobial susceptibility, virulence genes, and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia, China. J. Dairy Sci. 99:9560–9569. https://doi.org/10.3168/jds.2016-11625.

Wendlandt, S., J. Shen, K. Kadlec, Y. Wang, B. Li, W. J. Zhang, A. T. Feßler, C. Wu, and S. Schwarz. 2015. Multidrug resistance genes in staphylococci from animals that confer resistance to critically and highly important antimicrobial agents in human medicine. Trends Microbiol. 23:44–54. https://doi.org/10.1016/j.tim.2014.10.002.

Wilson, G.J., S.W. Tuffs, B.A. Wee, K.S. Seo, N. Park, T. Connelley, C.M. Guinane, W.I. Morrison, and J.R. Fitzgerald., 2018. Bovine *Staphylococcus aureus* superantigens stimulate the entire T cell repertoire of cattle. Infect. Immun. 86:e00505-18. https://doi.org/10.1128/iai.00505-18.

Wines, B. D., P. A. Ramsland, H. M. Trist, S. Gardam, R. Brink, J. D. Fraser, and P. M. Hogarth. 2011. Interaction of human, rat, and mouse immunoglobulin A (IgA) with staphylococcal superantigen-like 7 (SSL7) decoy protein and leukocyte IgA receptor. J. Biol. Chem. 286:33118–33124. https://doi.org/10.1074/jbc.M111.272252.

Wu, S., C. Piscitelli, H. de Lencastre, and A. Tomasz. 1996. Tracking the evolutionary origin of the methicillin resistance gene: Cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb. Drug Resist. 2:435–441. https://doi.org/10.1089/mdr.1996.2.435.

Wuytack, A., A. De Visscher, S. Piepers, F. Boyen, F. Haesebrouck, and S. De Vliegher. 2020. Distribution of non-*aureus* staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential. J. Dairy Sci. 103:10658–10675. https://doi.org/10.3168/jds.2020-18265.

Yaw, L. K., J. O. Robinson, and K. M. Ho. 2014. A comparison of long-term outcomes after meticillin-resistant and meticillin-sensitive *Staphylococcus aureus* bacteraemia: An observational cohort study. Lancet Infect. Dis. 14:967–975. https://doi.org/10.1016/S1473-3099(14)70876-X.

Paper III

Whole Genome Sequencing of Staphylococci Isolated from Bovine Milk Samples

M. Fergestad[1], F. Touzain[2], S. De Vliegher[4], A. De Visscher[4, 5], D. Thiry[3], C. Ngassam Tchamba[3, 6], J.G. Mainil[3], T. L'Abee-Lund[1], Y. Blanchard[2] and Y. Wasteson[1*]

[1]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 0454 Oslo, Norway

[2]Anses, Ploufragan/Plouzané/Niort Laboratory, Unit of Viral Genetics and Biosafety, Ploufragan, France

[3]Bacteriology, Department of Infection and Parasitic Diseases, Faculty of Veterinary Medicine, FARAH Research Centre, Liège University, 4000 Liège, Belgium

[4]M-team *and* Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium

[5]Present address: Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology and Food Science, Agricultural Engineering, Burg. Van Gansberghelaan 115 bus 1, 9820 Merelbeke, Belgium

[6]Present address: Vésale Pharma, Veterinary Department, Rue Louis Allaert 9, B-5310 Noville-sur-Mehaigne, Belgium

***Correspondence**:
Yngvild Wasteson
Yngvild.wasteson@nmbu.no

ABSTRACT

Staphylococci are among the commonly isolated bacteria from intramammary infections in bovines, where *Staphylococcus aureus* is the most studied species. This species carry a variety of virulence genes, contributing to bacterial survival and spread. Less is known about non-*aureus* staphylococci (NAS) and their range of virulence genes and mechanisms, but they are the most frequently isolated bacteria from bovine milk. Staphylococci can also carry a range of antimicrobial resistance genes, complicating treatment of the infections they cause. We used Illumina sequencing to whole genome sequence 93 staphylococcal isolates selected from a collection of staphylococcal isolates; 45 *S. aureus* isolates and 48 NAS isolates from 16 different species, determining their content of antimicrobial resistance genes and virulence genes. Antimicrobial resistance genes were frequently observed in the NAS species as a group compared to *S. aureus*. However, the lincosamide resistance gene *lnuA* and penicillin resistance gene *blaZ* were frequently identified in NAS, as well as a small number of *S. aureus*. The *erm* genes conferring macrolide resistance were also identified in several NAS isolates and in a small number of *S. aureus* isolates. In most *S. aureus* isolates, no antimicrobial resistance genes were detected, but in five *S. aureus* isolates three to six resistance genes were identified and all five of these carried the *mecA* gene. Virulence genes were more frequently identified in *S. aureus*, which contained on average five times more virulence genes compared to NAS. Among the NAS species there were also differences in content of virulence genes, such as *S. chromogenes* with a higher average number of virulence genes. By determining the content of a large selection of

virulence genes and antimicrobial resistance genes in *S. aureus* and 16 different NAS species our results contribute with knowledge regarding the genetic basis for virulence and antimicrobial resistance in bovine staphylococci, especially the less studied NAS. The results can create a broader basis for further research into the virulence mechanisms of this important group of bacteria in bovine intramammary infections.

Keywords: Non-*aureus* staphylolocci, *Staphylococcus aureus*, bovine, whole genome sequencing, antimicrobial resistance genes, virulence genes.

INTRODUCTION

The genus *Staphylococcus* includes a range of different species (De Buck et al., 2021) some of which are among the most commonly isolated bacteria causing intramammary infections in bovines (Pitkälä et al., 2004; Reksen et al., 2006; Olde Riekerink et al., 2008). For a long time *Staphylococcus aureus* has been the most recognized staphylococcal species causing both clinical and subclinical mastitis (Osterås et al., 2006; Olde Riekerink et al., 2008). The species is associated with a wide range of genes encoding a large diversity of virulence factors involved in adhesion, host immune evasion and biofilm formation (Foster et al., 2014; Geoghegan and Foster, 2017), toxins promoting inflammation and leukocyte death and exoenzymes cleaving and disabling immune molecules. All these factors contributing to bacterial survival, -spread and nutrient acquisition (Tam and Torres, 2019). However, in recent years, non-*aureus* staphylococci (NAS) have emerged as the most frequently isolated bacterial group from bovine milk in many countries and they are increasingly associated with intramammary infections (Pyörälä and Taponen, 2009; De Vliegher et al., 2012; Condas et al., 2017; De Buck et al., 2021).

Emergence of antimicrobial resistant staphylococci is of growing concern in the dairy industry. These bacteria can carry a large number of resistance determinants, which are often located on mobile genetic elements that facilitates horizontal spread of genes (Malachowa and DeLeo, 2010; Kadlec et al., 2012). NAS are regarded as a potential reservoir for antimicrobial resistance genes that can be transferred to and utilized by *S. aureus* (Otto, 2013; Vitali et al., 2014). Many NAS species are found as commensals on teat apices, hair, nares, vagina, teat and udder skin and inguinal skin, as well as in the environment in the barn (Taponen et al., 2008; Piessens et al., 2011; De Visscher et al., 2014), potentiating possible interactions with a variety of different bacteria present in the host. Due to these commensal properties, NAS may become exposed to several antimicrobials, not only by agents used for battling staphylococcal infections, but also by agents used to combat other pathogens (Kadlec et al., 2012). Several studies have pointed to some NAS species having a relatively greater impact on udder health, especially *S. chromogenes, S. simulans* and *S. xylosus* (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2019). However, contrary to *S. aureus*, the virulence factors of NAS, and mechanisms behind NAS' ability to colonize and infect the bovine udder are poorly described (Pyörälä and Taponen, 2009; Vanderhaeghen et al., 2015; Taponen et al., 2017). The extensive virulence gene profiling of 441 NAS isolates published by Naushad et al. (2019), the comparative study of 24 bovine-associated staphylococci of Åvall-Jääskeläinen et al. (2018) and the study on bovine NAS by Wuytack et al. (2020) are the most important published studies that have explored the virulence factors and

virulence mechanisms in bovine NAS species, which warrants further investigation of other NAS collections.

The aim of this study was therefore to collect more information on the genetic background for antimicrobial resistance and virulence in staphylococci from dairy cows. The objectives were i) whole genome sequencing of a collection of NAS and *S. aureus* isolated from bovine milk samples, and ii) determine the isolates' content of antimicrobial resistance and virulence genes, with emphasis on revealing the content of virulence genes in NAS compared to *S. aureus*.

MATERIALS AND METHODS

Collection and selection of isolates

The isolates originated from a previous study (Fergestad et al., 2021) of bovine staphylococci from Belgian and Norwegian dairy farms, in which 464 lactating cows were sampled from 13 farms in Belgium, and 100 cows with clinical mastitis were sampled from 100 farms in Norway (one cow from each farm). In that study, 272 staphylococcal isolates were characterized with regard to their phenotypic antimicrobial resistance, and carriage of the methicillin resistance genes *mecA* and *mecC* and some selected virulence genes were determined by PCR. From this collection, a total of 95 isolates were selected for whole genome sequencing (WGS) according to the following criteria: i) all *S. aureus* isolates , ii) representatives of all NAS species, iii) all isolates positive in PCR for *mecA* or *mecC*. Selection of NAS isolates was further based on phenotypic resistance patterns observed in species with more than one isolate, ensuring that both highly resistant and less resistant isolates were represented. Lastly, it was aimed to achieve an even distribution of isolates originating from the different geographical regions (Norway and Belgium). Altogether, the isolates selected for WGS were 45 *S. aureus* isolates and 50 NAS isolates [identified by Maldi Tof-MS; (Cameron et al., 2017; Cameron et al., 2018)] from 16 different species: *S. arlettae*, *S. auricularis*, *S. chromogenes*, *S. cohnii*, *S. devriesei*, *S. epidermidis*, *S. equorum S. haemolyticus*, *S. hominis*, *S. hyicus*, *S. saprophyticus*, *S. sciuri*, *S. simulans*, *S. vitulinus, S. warneri* and *S. xylosus*. Some reclassification within the genus *Staphylococcus* has recently been suggested; the reassignment of *S. sciuri* and *S. vitulinus* to a novel genus *Mammaliicoccus* (Madhaiyan et al., 2020) and the novel species *Staphylococcus pseudoxylosus*, closely related to *S. xylosus* (MacFadyen et al., 2019). For the sake of clarity, the suggested *Mammaliicoccus* species are considered as members of genus *Staphylococcus* and *S. pseudoxylosus* is included with the *S. xylosus* in this manuscript. The isolate identified as *S. pseudoxylosus* is, however, shown in the phylogenetic tree of NAS (Figure 1), to illustrate its phylogenetic placement.

DNA extraction, whole genome sequencing and assembly

DNA was extracted using Masterpure™ Gram Positive DNA Purification Kit (Lucigen, Middleton, USA). Quality and DNA concentrations were determined with Nanodrop 1000 (ThermoFisher Scientific).

The genomic DNA libraries were prepared for Illumina sequencing according to the manufacturer's instructions using the Nextera XT kit and sequenced by the NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA). The raw read sequences were assembled into contigs with the pipeline Shovill 1.0.4 (Seemann et al., 2020) including trimmomatic 0.38 (Bolger et al., 2014) for the cleaning and annotated using Prokka 1.13.3 (Seemann, 2014).

For LG-048 and LG-101 strains, Nanopore MinION long-read sequencing was performed using the Rapid Barcoding Sequencing kit (Oxford Nanopore) for library preparation. After guppy_gpu base calling, assembly of nanopore reads was performed using Canu 1.8 (Koren et al., 2017). The Illumina reads were cleaned with trimmomatic 0.36 (ILLUMINACLIP:illumina_oligos_and_revcomp:2:30:5:1:true LEADING:3 TRAILING:3 MAXINFO:40:0.2 MINLEN:36 options) and aligned with contigs provided by Canu assembly using BWA 0.7.15-r1140 (arXiv:1303.3997). Pilon 1.23 (Walker et al., 2014) was run on this alignment for preliminary corrections. The final result was the corrected consensus provided by Pilon.

The statistics of assemblies are provided in the Supplementary Table 1 of supplementary materials.

Phylogenetic trees

For creation of alignment and phylogenetic trees of the staphylococcal isolates we included some additional strains of different genera as outgroups to clarify where in the phylogenetic landscape the staphylococcal species were located. These strains were *Macrococcus bohemicus* (NZ_CM009972.1 to NZ_CM009973.1 and NZ_PZJG01000001.1 to NZ_PZJG01000029.1), *Abyssicoccus albus* (NZ_RKRK01000001.1 to NZ_RKRK01000010.1) and *Auricoccus indicus* (NZ_CP019573.1). We ran Panaroo 1.2.3 to analyze core genome to get common genes between the 95 strains (options '--clean-mode moderate –remove-invalid-genes -a core') and, using mafft in Panaroo, an alignment suitable for phylogenetic analysis. We picked two of the most common antimicrobial resistance genes in our material (*lnuA* and *blaZ*) and created phylogenetic trees of these genes to illustrate the phylogenetic relationship of the resistance genes across isolates and species. For the phylogenetic trees of antimicrobial resistance genes, we also used mafft in Panaroo for gene alignment. All phylogenetic trees were then created using IQtree 2.0.3 with core gene alignment (and options '--safe -T AUTO -B 1000 -alrt 1000 -m MFP'). The graphic representation of the phylogenetic trees was done with iTOL (Letunic and Bork, 2019).

Identification of antimicrobial resistance genes

Identification of antimicrobial resistance genes was performed with ResFinder 4.1 (Center for Genomic epidemiology, DTU) (Zankari et al., 2017; Bortolaia et al., 2020). Prokka 1.131.3 was used for detection of the multidrug efflux pump gene *norA*.

Identification of virulence genes

Identification of virulence genes was done by by using VirulenceFinder 2.0 (Center for Genomic Epidemiology, DTU) (Joensen et al., 2014) and a tblastn 2.10.1 search against a published dataset (Naushad et al., 2019). The dataset by Naushad et al. (2019) was used to complement the VirulenceFinder program which, unexpectedly, returned very few results from the NAS species. The tblastn search of the proteins in the Naushad et al. dataset was set upwith a minimum high-scoring segment pair (HPS) coverage $\geq$ 90, a minimum e-value of 10^{-5}. Ha scores were computed for all matches as described by Naushad et al. (2019). We kept only the hits with the highest Ha score and the highest percentage identity. Two proteins sequences were not identified in the database by Naushad et al. (2019): the phenol soluble modulin mec (PSMmec) and toxic shock syndrome toxin (tsst). For the PSMmec we used the record with GenBank accession number AIU84051.1, while the record for tsst from the database by Naushad et al. (2019) (accession number YP_415862) had been replaced by accession number WP_001035596.1, 100 % identical to the previous record over its full length. Both records were found searching the

NCBI (National Center for Biotechnology Information) website (https://www.ncbi.nlm.nih.gov/, accessed January 2021).

RESULTS

Staphylococcal isolates

Of the 95 isolates selected for WGS, results from 45 *S. aureus* and 48 NAS were included for further analysis. One *S. arlettae* and one *S. epidermidis* had to be rejected due to poor quality of the samples. The NAS species were divided into five different clades according to the phylogenetic analysis published by Naushad et al. (2016). NAS isolates were distributed in species and clades as follows; clade A was represented by *S. sciuri* (n=5) and *S. vitulinus* (n=1). Clade B was represented by *S. chromogenes* (n=6) and *S. hyicus* (n=1). Clade C consisted of *S. simulans* (n=2). Clade D was represented by *S. devriesei* (n=2), *S. epidermidis* (n=7), *S. haemolyticus* (n=9), *S. hominis* (n=4) and *S. warneri* (n=2). Clade E was represented by *S. arlettae* (n=1), *S. auricularis* (n=1), *S. cohnii* (n=2), *S. equorum* (n=2), *S. saprophyticus* (n=1) and *S. xylosus* (n=2). Species and clade distribution are also presented in Table 1-7. Figure 1 shows the phylogeny of the NAS isolates, confirming that they do separate according to the different clades. Figure 2 shows the phylogeny of the *S. aureus* isolates.

Antimicrobial resistance genes

Figure 1shows the distribution of antimicrobial resistance genes in all isolates and NAS species, in which the NAS are shown according to their clade.

Lincosamide resistance gene *lnuA* was present in 22 isolates from eight different species. Most NAS isolates originated from clade D (n=11). The gene was also present in *S. aureus*. Figure 3 shows the phylogeny of the *lnuA* genes, demonstrating that several species carried phylogenetically similar *lnuA* genes. Macrolide resistance genes were found in 17 different isolates, with some isolates (Ani-GT-049, Ani-LG-025, Ani-LG-026 and Ani-LG-125) containing multiple macrolide resistance genes. The *erm* genes were present in *S. aureus* (n=4) and, among the NAS, mainly species from clade D (n=5). The four *S. aureus* isolates contained the *ermA* gene, while the *ermB* and *ermC* genes were detected uniquely in some the NAS species. The *mphC* gene was present only in NAS species, most originating from clade E (n=4). The *msrA* gene was present in species from clade D and E.

The penicillin resistance gene *blaZ* was found in 19 isolates, both *S. aureus* and NAS, with most NAS isolates originating from clade D (n=11). Figure 4 shows the phylogeny of the *blaZ* genes. One *S. xylosus* isolate contained up to two phylogenetically distinct *blaZ* genes. The betalactam resistance gene *mecA* was present in seven isolates, *S. aureus* (n=5), and *S. epidermidis* (n=1) and *S. haemolyticus* (n=1) from clade D. Of these, four *S. aureus* and one *S. haemolyticus* also carried *blaZ*. All other resistance genes detected in *S. aureus* were detected in the *mecA* positive isolates, except for three non-*mecA S. aureus* isolates containing only the *lnuA* gene. Other variants of the *mec* genes were also detected in some isolates. All *S. sciuri* isolates (n=5) carried the *mecA1* gene, the only *S. vitulinus* carried the *mecA2* and one of the two *S. xylosus* carried *mecC2*.

Of the tetracycline resistance genes detectable by ResFinder, the *tetK* gene was present in 11 NAS isolates from clade A, D and E, and the *tetL* gene was present in three NAS species from clade B and D. The *tetM* gene was present in one *S. aureus* isolate only.

The aminoglycoside resistance gene *aadD* was identified in four *S. aureus* isolates and four NAS isolates consisting of the following species: *S. chromogenes*, *S. haemolyticus*, *S. sciuri* and *S. warneri*. One *S. aureus* isolate carried the aminoglycoside resistance gene *aac-aph*. The aminoglycoside resistance gene *str* was detected in nine NAS isolates from clade A, D and E.

Overall, in NAS, many antimicrobial resistance genes were found in isolates from clade D and most isolates in this clade carried one to three resistance genes. In one *S. chromogenes* isolate (clade B), seven resistance genes were detected, including *lnuA, blaZ, ermB, aadD, tetL, dfrK* and *fexA*. In addition, one *S. equorum*, one *S. cohnii* (both clade E) and one *S. warneri* (clade D) carried five resistance genes, with aminoglycoside, macrolide and tetracycline resistance genes detected in all three isolates. No antimicrobial resistance genes were identified in only nine of the 48 NAS isolates, which originated from clade B, C, D and E. On the other hand, no antimicrobial resistance genes were detected in the majority of the *S. aureus* isolates (37/45). Five *S. aureus* isolates carried three to six resistance genes, the *mecA* gene was detected in all five.

Using Prokka, the major facilitator superfamily multidrug efflux transporter gene *norA* was detected in all isolates, both *S. aureus* and NAS.

Virulence genes detected by VirulenceFinder

Virulence genes detected by VirulenceFinder (Center for Genomic Epidemiology, DTU) in the *S. aureus* isolates are shown in Figure 5. The *hlgA*, *hlgB* and *hlgC* genes encoding the gamma hemolysins A, B and C, and the *lukD* gene coding for a leukotoxin, were the most commonly detected virulence genes in *S. aureus* and detected in 44/45 *S. aureus* isolates. The *splB* gene, encoding a serine protease, and *aur*, encoding aureolysin, were detected in 43 and 41 *S. aureus* isolates, respectively. The least frequent virulence genes identified by VirulenceFinder were *sak*, *scn* and *sea*, encoding staphylokinase, staphylococcal complement inhibitor and enterotoxin A, respectively, found in two *S. aureus* isolates only (Ani-LG-017 and Ani-LG-027), and the *sed* and *seh* genes coding for enterotoxin D and H, were detected in one *S. aureus* isolate each.

Except for the detection of the *ACME* gene in four *S. epidermidis* isolates, the VirulenceFinder did not detect any other virulence genes in the NAS isolates.

Putative virulence factors based on dataset by Naushad et al.

Virulence factors involved in adherence

Based on the dataset by Naushad et al. (2019) 28 virulence factors involved in adherence were tested. The genes are listed, and results are summarized in Table 1. The *atl* gene was present in 21 NAS isolates and in six of the 16 NAS species from clade A, B, and D, as well as all *S. aureus* isolates. The *icaA* of the *ica* operon was present in six NAS isolates from clade A and E. The *icaC, icaD* and *icaR* were detected in three, two and one NAS isolates respectively, from clade E. Regarding the *S. aureus* isolates, *icaB*, *icaC, icaD* and *icaR* were present in all isolates,

while *icaA* was present in 41 isolates. Many of the adherence associated genes were not detected in either NAS species isolates nor *S. aureus* isolates, including *aap, bap, clfA* and *clfB, ebp, uafA*, *fnbA* and *fnbB, sasG* and *sasp* and the Ser-Asp rich fibrinogen-binding proteins.

Exoenzymes

Twenty-one different exoenzyme genes were tested. The genes are listed, and results are summarized in Table 2. The *nuc* gene was present in 36 out of 48 NAS isolates in species from clade B, C, D and E. Only one *S. hyicus* isolate was positive in clade B, while in clade C, D and E all isolates were positive. The second most frequent exoenzyme genes in NAS were *aur* and *geh*, detected in 27 and 24 isolates, respectively. The *aur* gene was detected in 10 NAS species from clade A, B, D and E, while the *geh* gene was identified in seven NAS species from clade C, D and E. Nine of the *geh*-positive NAS isolates were also positive for *lip*. The *sspB* gene was detected in 12 NAS isolates from clade B, C and D and the *sspC* was also present in two *S. warneri* isolates (clade D). Among the serine proteases, *splC* and *splE* were detected in *S. xylosus* and *S. chromogenes*, respectively and *vWpb* was found in *S. chromogenes*. Many of the exoenzymes were not detected in NAS species. For *S. aureus*, the exoenzymes *adsA*, *sspB* and *sspC*, *lip*, *geh*, *coa* and *nuc* were identified in all isolates, while *hysA* was detected in all but one, and *aur*, *splA-F* and *vWbf* were detected in 43, 43 and 42 of the 45 *S. aureus* isolates, respectively. This means that 93 % of all *S. aureus* isolates contained at least 16 exoenzyme genes. The *sak* gene was identified in two *S. aureus* isolates.

Virulence factors involved in host immune evasion

Twenty virulence genes associated with host immune evasion were tested. The genes are listed, and results are summarized in Table 3. The *chp*, *spa* and *sbi* genes were not detected in any of the NAS species. The *scn* gene was only identified in species from clade B. The *cap* genes were the most frequently detected host immune evasion genes. Based on Naushad et al. (2019) *cap* genes were considered present if either the *cap5* or *cap8* isoforms were detected. The *capP* was detected in all NAS isolates and five NAS species contained only this *cap* gene. Two *S. haemolyticus* isolates contained all 16 *cap* genes and the single *S. hyicus* and *S. arlettae* contained 14 *cap* genes. All *cap* genes except *capD* were present in all *S. aureus* isolates. The *scn*, *spa* and *sbi* genes were identified in 44, 34 and 43 *S. aureus* isolates, respectively. The *chp* gene was not detected in any of the *S. aureus* isolates.

Virulence factors involved in iron uptake and metabolism

Twenty-nine iron uptake and metabolism genes were tested. The genes are listed, and results are summarized in Table 4. Of the iron-regulated surface determinant genes, *isdF* was present in 36 NAS isolates and in 13 NAS species from all clades, *isdI* was detected in 34 isolates and in 14 different NAS species and *isdG* was detected in 30 isolates of 11 different NAS species. The ABC transporter genes and staphyloferrin A and B synthesis related genes were most frequently identified in NAS isolates, where *htsC* and *sbnA* were present in all NAS isolates. All genes associated with iron uptake and metabolism were detected in all *S. aureus* isolates.

Toxins, type VII secretion and phenol-soluble modulin genes

The genes in this category included 36 toxin genes from various categories. The genes are listed, and results are summarized in Table 5. The enterotoxins and staphylococcal exotoxins are described below and in Tables 6 and 7. Most of the toxin genes in this category were not detected in the NAS species, except *etc* which was present in all NAS isolates, *hlb* present in all *S. epidermidis* isolates and *etb* present in all *S. sciuri* isolates. The type VII secretion genes *esaA*, *esaB*, *essA*, *essC* and *esxA* were identified in three NAS isolates, namely one *S. chromogenes*, one *S. epidermidis* and the single *S. hyicus*. Among the PSMs, none of the PSMα were detected, while the PSMβ genes were present in all NAS species except the species in clade A where PSMmec was identified. As for the *S. aureus* isolates, all isolates contained *hla*, *hlgA, hlgB, hlgC* and all leukocidin and leukotoxin genes. The *etc* gene was identified in 44 isolates and the *tsst* and *hld* genes were identified in 13 and 11 *S. aureus* isolates, respectively, while none of the *S. aureus* isolates contained *hlb*, *eta, etb* or *etd*. Most of the type VII secretion system genes were frequently detected in *S. aureus* isolates, except *essB* that was not present in any of the isolates. Of the PSMs, *PSMα1* and *PSMβ1* to *PSMβ6* were identified in all *S. aureus* isolates, the remaining PSMs were not identified.

Thirty-six staphylococcal exotoxins (SETs) and 21 enterotoxins were tested. Except for the species in clade B, *set* genes were not detected in any NAS species. The enterotoxins were only identified in the single *S. hyicus* of clade B, this isolate contained all enterotoxin genes except *sej* and *yent1*. In the *S. aureus* isolates most *set* genes were present in all isolates, *set8, set9, set19, set 32, set33* and *set38* were frequently detected, but not present in all isolates, while *set2* was only present in one *S. aureus* isolate. . The *yent2* gene was detected in all *S. aureus* isolates. The second most frequently identified enterotoxin genes were *sea*, *selo* and *selp*, present in 28, 23 and 23 *S. aureus* isolates. The *sej* gene was not detected in any *S. aureus* isolates.

Virulence potential

Virulence potential was defined as the total number of virulence genes in an isolate, where all genes were equally weighted (Naushad et al., 2019). *Staphylococcus aureus* had the highest virulence potential and carried on average 140 virulence genes. The NAS isolates, disregarding species, carried on average 28 virulence genes. The highest virulence potential in NAS was detected in *S. chromogenes* and *S. hyicus* (both clade B). *Staphylococcus chromogenes* isolates contained on average 44 virulence genes, and, except the single *S. hyicus* isolates, was the only NAS species were staphylococcal exotoxins were detected. The single *S. hyicus* isolate contained 98 virulence genes, carrying both exotoxins and enterotoxins. The lowest virulence potential was found in *S. sciuri* and *S. hominis* with, on average, 13 and 21 virulence genes in total, respectively.

DISCUSSION

This study of virulence and antimicrobial resistance genes in 93 whole-genome sequenced NAS and *S. aureus* isolates from bovine milk samples of European origin adds new data to the current sparse information of the genetic basis for both antimicrobial resistance and virulence factors in

bovine staphylococci. WGS confirmed the species distribution previously determined with Maldi Tof-MS of the isolate collection of 45 *S. aureus* and 48 NAS isolates of different species (Fergestad et al. 2021). In total, we determined the presence of 191 staphylococcal virulence genes and 25 antimicrobial resistance genes, unevenly distributed among NAS and *S. aureus*, but also among the NAS species themselves. One main finding is that our description of the virulence gene contents of the 48 NAS isolates coincides to a large extent with the findings of the so far most comprehensive study of virulence genes in a collection of Canadian bovine NAS based on WGS data. Further interpretations of our main findings are discussed below.

The emergence and spread of antimicrobial resistance genes are of great concern to society, including animal food production and the dairy industry. The antimicrobial resistance genes that are detected by ResFinder 4.1 were frequently observed in our collection of isolates representing 16 NAS species, coinciding with other reports of occurrence of such genes distributed among several NAS species (Nobrega et al., 2018). This finding of an array of resistance genes in a diversity of NAS species is supporting the hypothesis that these bacteria can act as a potential reservoir for resistance properties (Otto, 2013; Becker et al., 2014). It is, however, noteworthy that although several resistance genes were also observed in *S. aureus*, these genes were mainly associated with the few MRSA isolates of the collection (see below).

We found several isolates from different staphylococcal species harboring phylogenetically similar *lnuA* genes. This gene, encoding a lincosamide nucleotidyltransferase that confers resistance to lincosamides, has previously been found in both *S. aureus* and NAS of bovine origin (Lüthje and Schwarz, 2006; Li et al., 2015). The gene is often found on plasmids, which could promote horizontal transfer of the gene (Lüthje et al., 2007). Studies have shown that the nucleotide sequence of *lnuA* is more conserved than the surrounding plasmid sequences and the conserved gene has been found in several different plasmid backbones, suggesting that the gene is also exchanged via interplasmid recombinational events (Lüthje et al., 2007; Wassenaar et al., 2016). This may contribute to explaining our finding of phylogenetically similar *lnuA* genes in several different staphylococcal species.

Resistance to betalactam antimicrobials is commonly reported in staphylococci. The *blaZ* gene encodes a penicillinase (or betalactamase) conferring penicillin resistance by hydrolyzing the betalactam ring and inactivating the drug (Zhang et al., 2001). The gene is usually either plasmid- or chromosomally encoded (Olsen et al., 2006). Penicillin resistance is prevalent in *S. aureus* of both human and bovine origin (Olsen et al., 2006) and betalactamase production is the most prevalent mechanism of betalactam resistance in NAS (Nobrega et al., 2018) . In consistence with these observations, we found several isolates of different staphylococcal species carrying the *blaZ* gene. The phylogenetic tree of the gene also showed several phylogenetically different sequences. A high number of different *blaZ* sequence types has previously been shown in staphylococci of bovine origin, as well as a very low similarity between plasmid- and chromosomally encoded *blaZ* genes which, in a study by Olsen et al. (2006), separate into two phylogenic clusters, leading to the conclusion that exchange of *blaZ* between plasmid and chromosome and between strains are rare events (Olsen et al., 2006). Our *blaZ* phylogenetic tree (Fig 4) also display two separate clusters. Interestingly, we observed one *S. xylosus* isolate with two distinct *blaZ* genes, one from each branch/cluster.

We identified five *mecA* positive *S. aureus* isolates, of which four were from the same herd and epidemiologically related. The *mecA*-positive *S. aureus* isolates clearly differed from the rest of

the *S. aureus* isolates, with a larger content of antimicrobial resistance genes. A higher frequency of antimicrobial resistance in methicillin-resistant *S. aureus* (MRSA) compared to methicillin-susceptible *S. aureus* (MSSA) has been shown in human isolates (Thompson and Brown, 2014). Two NAS isolates also carried the *mecA* gene, one being a *S. epidermidis*. *Staphylococcus epidermidis* has previously been shown to have a higher prevalence of *mecA* compared to other NAS species (Nobrega et al., 2018). In addition, all *S. sciuri* harbored the *mecA1* gene, while the *mecA2* and *mecC2* were found in one *S. vitulinus* and one *S. xylosus*, respectively. There is support for a theory suggesting that *mecA* evolved from native *mec* genes in species of the *S. sciuri* group (Couto et al., 1996; Zhou et al., 2008; Antignac and Tomasz, 2009). The *mecA1* gene, thought to be ubiquitous in *S. sciuri*, is believed to be the most ancestral form of *mecA* and shares 85 % nucleotide identity with *S. aureus mecA*, while the *mecA2* of *S. vitulinus* is an intermediate form with 94 % homology (Miragaia, 2018). However, neither *mecA1* or *mecA2* generally confers methicillin resistance (Couto et al., 1996; Wu et al., 1996; Miragaia, 2018). The *mecC2* gene, a *mecC* allotype with 92.9% identity with the *S. aureus mecC*, has previously been described in *S. saprophyticus* (Małyszko et al., 2014).

Several staphylococcal species in our study also harbored the macrolide resistance genes *ermA-C*. In the *S. aureus* isolates only *ermA* was found, while *ermC* and to some extent *ermB* were found in the NAS species. The *ermA*-positive *S. aureus* isolates were also *mecA* positive, labeling them as MRSA. This concurs with the results of a previous study that found *ermA* to be more prevalent than *ermB* and *ermC* in MRSA isolates and the *ermC* to be more prevalent than *ermA* and *ermB* in NAS (Lina et al., 1999).

Several studies have supported the role of drug efflux in the development of antimicrobial resistance in *S. aureus* (DeMarco et al., 2007; Kwak et al., 2013; Santos Costa et al., 2015). Especially multidrug efflux pumps are of interest, being able to remove several chemically different substances and often linked to multidrug resistant phenotypes (Piddock, 2006; Poole, 2007). The major facilitator superfamily multidrug efflux transporter norA is the best studied efflux system in *S. aureus* and is associated with resistance to fluoroquinolones and several antiseptics and disinfectants (Costa et al., 2018). The gene is believed to be a part of the core genome of *S. aureus* (Costa et al., 2018), while Nobrega et al. (2018) reported the *norA* gene in 91 % of NAS isolates. Consequently, our finding of the *norA* gene in all isolates, both in *S. aureus* and NAS, is coherent with these previous data.

Many antimicrobial resistance genes were detected in NAS isolates from clade D. Multidrug resistant *S. epidermidis* and *S. haemolyticus*, both from clade D, have previously been isolated from both humans and animals (Anthonisen et al., 2002; Lee et al., 2018; Nobrega et al., 2018). However, it should be noted that this clade was also the clade with the highest number of isolates, potentially skewing the results.

Regarding virulence there are many factors involved in colonization, infection, and bacterial survival. Adhesion is one of the first steps leading to colonization and infection and the process is also needed for biofilm formation. We analyzed the genome data for the presence of 28 adherence and biofilm associated virulence factor genes. The *atl* gene, most frequently observed in NAS, is involved in biofilm formation through initiating adherence, followed by production of polysaccharide intracellular adhesins encoded by the *ica*-operon, forming a polysaccharide-based biofilm (Naushad et al., 2019). Deviating results about the distribution of *ica* genes in bovine NAS have been reported (Piessens et al., 2012; Tremblay et al., 2013), and a study concerning

the *icaA* genes of nine food-related NAS species showed considerable sequence diversity between strains of the same species (Møretrø et al., 2003). Diverging sequences could explain differences in the detection of *ica* genes. Our observation of the frequent detection of *icaA* followed by *icaC* and *icaD* resembles the findings of Naushad et al. (2019) who found *icaC*, followed by *icaA* and *icaD* to be most frequent. The genes in the *ica* operon were detected in all *S. aureus* isolates in this study, except *icaA* which was identified in 91 %, similar to findings from other studies (Melchior et al., 2009; Melchior et al., 2011). In human staphylococcal strains, biofilms associated with the *ica*-operon are often related to infections in foreign devices, leading to the hypothesis that the *ica* genes in bovine isolates could play a role outside the udder, by promoting adhesion to abiotic surfaces, such as milking equipment (Melchior et al., 2011).

The production of exoenzymes further facilitates colonization and infection. Following adhesion, exoenzymes contributes to disable the host immune system, damage tissue and acquire nutrients (Tam and Torres, 2019). The thermonuclease gene *nuc* was the most frequently observed exoenzyme gene in NAS species in our study, consistent with recent studies (Åvall-Jääskeläinen et al., 2018; Naushad et al., 2019). The *aur* gene and both lipase gene *geh* and *lip* were frequently observed in the NAS group, however, the lipase genes only in clades C, D and E. These results also concur with the findings by Naushad et al. (2019), who found these genes to be frequently distributed in clades B to E. In addition, similar to Naushad et al. (2019), we detected *vWbp* in *S. chromogenes*, which could explain the variable coagulase test results for this species (Dos Santos et al., 2016). A large proportion of the *S. aureus* isolates in this study contained most of the tested exoenzymes. It is well known that *S. aureus* can produce a vast variety of exoenzymes, degrading host and bacterial molecules to escape the host immune system and gain nutrients, contributing to the success of the pathogen (Tam and Torres, 2019).

Staphylococci, especially *S. aureus*, have several host immune evasion virulence factors, such as genes allowing production of capsular polysaccharides, enabling bacterial survival and dissemination by hindering phagocytosis and increasing virulence (Kuipers et al., 2016). The cap5A-P and cap8A-P are prevalent in *S. aureus* of bovine origin (Salimena et al., 2016). In our study, *capP* was present in all NAS isolates, while *capA-D*, *capM* and *capO* were present in almost half of the NAS isolates. This deviates some from the findings of Naushad et al. (2019), who found *capM* to be most frequent and *capA-L* in low frequencies. Except *capD*, all *cap* genes were present in all *S. aureus* isolates, however, *capD* was also present in most *S. aureus* isolates.

In addition to the capsular genes, staphylococci can produce other important immune evasion virulence factors, such as *chp*, *scn*, *spa* and *sbi*. The chemotaxis inhibitory protein (encoded by *chp*) and staphylococcal complement inhibitor (encoded by *scn*) are mostly believed to be found in staphylococci from human sources (Verkaik et al., 2011). Consistent with this finding, we did not detect the *chp* gene in any isolate. However, in accordance with the study by Naushad et al. (2019), we identified *scn* in species of clade B. The *scn* gene was also detected in all except one of the *S. aureus* isolates.

Iron is an essential micronutrient involved in several metabolic processes, vital for bacterial survival and growth (Sheldon and Heinrichs, 2015). During infection, the host withdraws free iron from body fluids to suppress pathogens (Haley and Skaar, 2012; Sheldon and Heinrichs, 2015). Mechanisms to acquire iron in a situation where the supply is scarce are well studied in *S. aureus*, who can take up iron directly from molecules using *isd* genes and produce siderophores along with surface transporters (Sheldon and Heinrichs, 2015). We did indeed

detect all iron uptake and metabolism genes, except *isdA*, in all *S. aureus* isolates. In the NAS species, however, ABC transporter and staphyloferrin A genes were more frequently detected, compared to *isd* genes and staphyloferrin B genes. This is in accordance with the study by Naushad et al. (2019), and supports their hypothesis that staphyloferrin A production is the principal mechanism for iron acquisition in NAS.

The production of toxins is another important determinant of virulence in staphylococci, especially in *S. aureus*. These toxins, such as cytotoxins (hemolysins, leukotoxins and leukocidins) and superantigens (enterotoxins, exfoliative toxins and toxic shock syndrome toxins (TSST)), promotes inflammation and leukocyte cell death (Tam and Torres, 2019). Of the cytotoxins, we detected *hlb*, encoding beta hemolysin, in all *S. epidermidis* isolates (clade D), similar to Naushad et al. (2019), who found *hlb* to be the most frequent hemolysin. Surprisingly, we did not detect *hlb* in any of the *S. aureus* isolates, whereas *hla* was present in all isolates. This contrasts the findings from a study on bovine and humans *S. aureus* isolates where *hlb* appeared more common in bovine *S. aureus* isolates, while *hla* was more prevalent in the human isolates (Aarestrup et al., 1999). Similar to the study by Naushad et al. (2019), we did not detect any leukocidin genes or leukotoxin genes in the NAS isolates. Åvall-Jääskeläinen et al. (2018) found *lukD* in one *S. simulans* isolate, however, none of the other leukocidin or leukotoxin genes were detected in NAS in their study. All the *S. aureus* isolates in our study contained all genes for leukocidins and leukotoxins, including *lukS-PV* and *lukF-PV*. This resembles findings from another study on bovine *S. aureus* isolates that found leukocidin and leukotoxin genes in most isolates. However, that study did not detect any isolates carrying *lukS-PV* (Yamada et al., 2005). The Panton Valentine Leukocidin (PVL) genes are believed to be restricted to human strains of *S. aureus* (Vrieling et al., 2016) and it was surprising to find these genes in all our bovine *S. aureus* isolates. It is possible that this result appeared due to the method of similarity search, as there is a possibility for detecting genes that are similar to the gene in question, although not being the same gene. This could explain our results, as many of the *lukS-PV* and *lukF-PV* genes in our study had the same percentage identity in several *S. aureus* isolates, possibly indicating that there are sequence similarities between the genome and the genes, although the genes in question are not actually present. However, this warrants further investigation. The PVL genes have been reported in a few NAS isolates of bovine origin in India (Mahato et al., 2017). Of the exfoliative toxin genes, we detected *etc* in all NAS isolates and *etb* in all *S. sciuri* isolates. This contrasts the findings of Naushad et al. (2019) who found *eta* in all isolates of three NAS species and *etb* in a few isolates of *S. agnetis* and Åvall-Jääskeläinen et al. (2018) who also found *etb* in *S. agnetis*. However, the latter did not test for *eta, etc* and *etd*. We did not have any *S. agnetis* in our collection, and it is unknown whether there are geographical differences between the distribution of the different exfoliative toxin genes or if there are other factors affecting our results. Of the exfoliative toxin genes, only the *etc* gene was detected in *S. aureus* isolates. The lack of *eta* and *etb* has been shown previously by Haveri et al. (2007), however, these were the only two exfoliative toxin genes included in this study. The *eta* gene has been detected in a few *S. aureus* isolates from bovines (Hayakawa et al., 2001).

Phenol soluble modulins (PSM) are also involved in the killing of leukocytes and can act synergistically with leukocidins (Hongo et al., 2009), contributing to the leukotoxicity of *S. aureus*, as well as being involved in biofilm formation (Otto, 2014; Vrieling et al., 2016). The PSMs are considered major determinants of the virulence of *S. aureus* and α-type PSMs are thought to be more aggressive than β-type PSMs (Otto, 2014; Naushad et al., 2019). Being

encoded on the core genome, the PSMs are present in virtually all staphylococci (Otto, 2014). We detected β-type PSMs genes, encoding the least aggressive PSMs, in most NAS species, but not in species of clade A and the *S. arlettae* isolate (clade E). This concurs with the results reported by Naushad et al. (2019). However, in the species of clade A (*S. sciuri* and *S. vitulinus*) we detected the *PSMmec* gene. This is the only exception to the core genome-encoded PSMs, as the *PSMmec* is often found on the SCC*mec* cassette carrying the *mecA* genes conferring methicillin resistance (Qin et al., 2016). The isolates in our study in which the *PSMmec* was found did indeed harbor *mec* genes, as the *S. sciuri* isolates harbored the *mecA1* and the *S. vitulinus* isolate harbored the *mecA2* gene. The *PSMmec* has been identified in *S. vitulinus* carrying SCC*mec* previously (Monecke et al., 2012). In *S. aureus PSMα1*, as well as *PSMβ1-6* were detected in all isolates. The finding of α-type PSM in *S. aureus* and not in NAS can be due to the aggressive potential of *S. aureus*, as the α-type PSM are considered more aggressive and this type of PSMs is mostly associated with *S. aureus* (Wang et al., 2007). The fact that we only detected *PSMα1* and not the other *PSMα* can be due to limitations in the method used (similarity search) and the short sizes of *PSMα*, as this often does not give meaningful results (Cheung et al., 2014; Otto, 2014).

Superantigens are responsible for much of the toxicity in staphylococci. They are robust toxins, resilient to heat, proteolysis and desiccation (Spaulding et al., 2013; Tam and Torres, 2019). Toxic shock syndrome toxin gene (*tsst*) was not detected in the NAS isolates in our study. This concurs with several previous studies (Xu et al., 2015; Mello et al., 2016; Naushad et al., 2019). The *tsst* gene is mostly associated with *S. aureus* and has been detected in bovine *S. aureus* previously (Artursson et al., 2016; Vaughn et al., 2020). We only detected staphylococcal exotoxins in species of clade B (*S. hyicus* and *S. chromogenes*), however, several exotoxins were present in all *S. aureus* isolates. We only detected enterotoxin genes in one *S. hyicus* isolates (clade B). Naushad et al. (2019) also detected enterotoxin genes only in species from clade B. However, unlike Naushad et al. (2019) we did not identify any enterotoxin genes in *S. chromogenes*. We also detected several enterotoxin genes in *S. aureus* isolates. The *S. aureus* enterotoxins can cause acute and severe food poisoning, making it important to avoid contamination of enterotoxin-producing *S. aureus* isolates throughout the food production chain (Nia et al., 2016). Raw milk and cheese made of unpasteurized milk are well-known food sources of food poisoning caused by *S. aureus*.

Regarding the total virulence gene content, *S. aureus* stands out from NAS with, on average, five times as many virulence genes as NAS, highlighting the large virulence potential of *S. aureus* and the limited virulence potential of NAS. When looking at the total number of virulence genes in NAS, *S. chromogenes* had a higher virulence potential than most other species, mostly due to the presence of exotoxins. The single *S. hyicus* isolate stood apart with the highest virulence potential, due to host immune evasion, exotoxin and enterotoxin genes. This concurs with the findings of Naushad et al. (2019) who also found the highest virulence potential in species from clade B, including *S. chromogenes* and *S. hyicus*. Virulence is dependent on context and is often characterized by an intricate interplay between the microorganism and the host, making it difficult to predict virulence based on virulence gene content alone (Balloux et al., 2018). Nor is it a given that the virulence genes are expressed in the microorganism, even if they are present (Chaves-Moreno et al., 2016), which further complicates the matter. Considering the apparent complexity of virulence and the limited knowledge on the subject in bovine NAS, further studies on the association between virulence and clinical impact of these species is important. As several

studies indicates that *S. chromogenes* could have a greater impact on udder health (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2019) and both this study and Naushad et al. (2019) found *S. chromogenes* among those with the highest virulence potential, further studies of the virulence and pathogenesis of this species should be emphasized (De Buck et al., 2021).

The results from VirulenceFinder supported the results from the database by Naushad et al. (2019) regarding the *S. aureus* isolates. However, some genes were not detected as frequently with the VirulenceFinder, possibly due to stricter thresholds for identity in VirulenceFinder. In addition, the VirulenceFinder returned less virulence genes compared to the database from Naushad et al. (2019). This could be because VirulenceFinder contains less virulence genes compared to the database by Naushad et al. (2019), as the database was expanded by doing blast similarity search to identify genes. Although this method does identify true genes, there is a possibility for misinterpretations due to detection of similar, but not identical genes.

In conclusion, this study shows that there are more antimicrobial resistance genes in NAS compared to *S. aureus*, with the exception of MRSA. Regarding virulence, *S. aureus* has a higher virulence potential compared to NAS, but there are also differences in virulence gene content within the NAS group.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. **FT** was in charge of the methodology and performed the methods used. **MF** organized the databases and analyzed the results. **SV, AV** and **YW** contributed to the analysis of the results. **MF** wrote the first draft of the manuscript. **FT** wrote sections of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

DATA AVAILABILITY

All genomic data related to this project, including raw reads, are available via the NCBI BioProject PRJNA609060.

FUNDING

This project was part of the ERA-Net Animal Health and Welfare (ANIHWA), MRSA_bacteriophages project. The project was funded by the Research Council of Norway, the Research Foundation – Flanders (FWO), and Service Public Fédéral – Santé publique, Sécurité de la Chaîne alimentaire et Environnement (Wallonia). Salary was provided by each home institution.

REFERENCES

Aarestrup, F.M., Larsen, H.D., Eriksen, N.H., Elsberg, C.S., Jensen, N.E., 1999. Frequency of alpha- and beta-haemolysin in Staphylococcus aureus of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. Apmis. 107, 425-430.

598 Anthonisen, I.L., Sunde, M., Steinum, T.M., Sidhu, M.S., Sørum, H., 2002. Organization of the
599 antiseptic resistance gene qacA and Tn552-related beta-lactamase genes in multidrug-
600 resistant Staphylococcus haemolyticus strains of animal and human origins. Antimicrob
601 Agents Chemother. 46, 3606-3612. https://doi.org/10.1128/aac.46.11.3606-3612.2002.
602 Antignac, A., Tomasz, A., 2009. Reconstruction of the phenotypes of methicillin-resistant
603 Staphylococcus aureus by replacement of the staphylococcal cassette chromosome mec
604 with a plasmid-borne copy of Staphylococcus sciuri pbpD gene. Antimicrob Agents
605 Chemother. 53, 435-441. https://doi.org/10.1128/aac.01099-08.
606 Artursson, K., Söderlund, R., Liu, L., Monecke, S., Schelin, J., 2016. Genotyping of
607 Staphylococcus aureus in bovine mastitis and correlation to phenotypic characteristics.
608 Vet Microbiol. 193, 156-161. https://doi.org/10.1016/j.vetmic.2016.08.012.
609 Balloux, F., Brønstad Brynildsrud, O., van Dorp, L., Shaw, L.P., Chen, H., Harris, K.A., Wang,
610 H., Eldholm, V., 2018. From Theory to Practice: Translating Whole-Genome Sequencing
611 (WGS) into the Clinic. Trends Microbiol. 26, 1035-1048.
612 https://doi.org/10.1016/j.tim.2018.08.004.
613 Becker, K., Heilmann, C., Peters, G., 2014. Coagulase-negative staphylococci. Clin Microbiol
614 Rev. 27, 870-926. https://doi.org/10.1128/cmr.00109-13.
615 Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina
616 sequence data. Bioinformatics. 30, 2114-2120.
617 https://doi.org/10.1093/bioinformatics/btu170.
618 Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A.,
619 Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann,
620 B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B.B.,
621 Malhotra-Kumar, S., Westh, H., Pinholt, M., Anjum, M.F., Duggett, N.A., Kempf, I.,
622 Nykäsenoja, S., Olkkola, S., Wieczorek, K., Amaro, A., Clemente, L., Mossong, J.,
623 Losch, S., Ragimbeau, C., Lund, O., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions
624 of phenotypes from genotypes. J Antimicrob Chemother. 75, 3491-3500.
625 https://doi.org/10.1093/jac/dkaa345.
626 Cameron, M., Barkema, H.W., De Buck, J., De Vliegher, S., Chaffer, M., Lewis, J., Keefe, G.P.,
627 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-
628 assisted laser desorption/ionization time-of-flight mass spectrometry using a direct
629 transfer protocol. J Dairy Sci. 100, 2137-2147. https://doi.org/10.3168/jds.2016-12020.
630 Cameron, M., Perry, J., Middleton, J.R., Chaffer, M., Lewis, J., Keefe, G.P., 2018. Short
631 communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference
632 spectra expanded database for the identification of bovine-associated coagulase-negative
633 staphylococci. J Dairy Sci. 101, 590-595. https://doi.org/10.3168/jds.2017-13226.
634 Chaves-Moreno, D., Wos-Oxley, M.L., Jáuregui, R., Medina, E., Oxley, A.P., Pieper, D.H.,
635 2016. Exploring the transcriptome of Staphylococcus aureus in its natural niche. Sci Rep.
636 6, 33174. https://doi.org/10.1038/srep33174.
637 Cheung, G.Y., Joo, H.S., Chatterjee, S.S., Otto, M., 2014. Phenol-soluble modulins--critical
638 determinants of staphylococcal virulence. FEMS Microbiol Rev. 38, 698-719.
639 https://doi.org/10.1111/1574-6976.12057.
640 Condas, L.A.Z., De Buck, J., Nobrega, D.B., Carson, D.A., Naushad, S., De Vliegher, S.,
641 Zadoks, R.N., Middleton, J.R., Dufour, S., Kastelic, J.P., Barkema, H.W., 2017.
642 Prevalence of non-aureus staphylococci species causing intramammary infections in

643 Canadian dairy herds. J Dairy Sci. 100, 5592-5612. https://doi.org/10.3168/jds.2016-
644 12478.
645 Costa, S.S., Sobkowiak, B., Parreira, R., Edgeworth, J.D., Viveiros, M., Clark, T.G., Couto, I.,
646 2018. Genetic Diversity of norA, Coding for a Main Efflux Pump of Staphylococcus
647 aureus. Front Genet. 9, 710. https://doi.org/10.3389/fgene.2018.00710.
648 Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J.A., Hubner, R.J., Sanches, I.S.,
649 Tomasz, A., 1996. Ubiquitous presence of a mecA homologue in natural isolates of
650 Staphylococcus sciuri. Microb Drug Resist. 2, 377-391.
651 https://doi.org/10.1089/mdr.1996.2.377.
652 De Buck, J., Ha, V., Naushad, S., Nobrega, D.B., Luby, C., Middleton, J.R., De Vliegher, S.,
653 Barkema, H.W., 2021. Non-aureus Staphylococci and Bovine Udder Health: Current
654 Understanding and Knowledge Gaps. Frontiers in Veterinary Science.
655 8https://doi.org/10.3389/fvets.2021.658031.
656 De Visscher, A., Supré, K., Haesebrouck, F., Zadoks, R.N., Piessens, V., Van Coillie, E.,
657 Piepers, S., De Vliegher, S., 2014. Further evidence for the existence of environmental
658 and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet
659 Microbiol. 172, 466-474. https://doi.org/10.1016/j.vetmic.2014.06.011.
660 De Vliegher, S., Fox, L.K., Piepers, S., McDougall, S., Barkema, H.W., 2012. Invited review:
661 Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J
662 Dairy Sci. 95, 1025-1040. https://doi.org/10.3168/jds.2010-4074.
663 DeMarco, C.E., Cushing, L.A., Frempong-Manso, E., Seo, S.M., Jaravaza, T.A., Kaatz, G.W.,
664 2007. Efflux-related resistance to norfloxacin, dyes, and biocides in bloodstream isolates
665 of Staphylococcus aureus. Antimicrob Agents Chemother. 51, 3235-3239.
666 https://doi.org/10.1128/aac.00430-07.
667 Dos Santos, D.C., Lange, C.C., Avellar-Costa, P., Dos Santos, K.R., Brito, M.A., Giambiagi-
668 deMarval, M., 2016. Staphylococcus chromogenes, a Coagulase-Negative
669 Staphylococcus Species That Can Clot Plasma. J Clin Microbiol. 54, 1372-1375.
670 https://doi.org/10.1128/jcm.03139-15.
671 Foster, T.J., Geoghegan, J.A., Ganesh, V.K., Höök, M., 2014. Adhesion, invasion and evasion:
672 the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol.
673 12, 49-62. https://doi.org/10.1038/nrmicro3161.
674 Fry, P.R., Middleton, J.R., Dufour, S., Perry, J., Scholl, D., Dohoo, I., 2014. Association of
675 coagulase-negative staphylococcal species, mammary quarter milk somatic cell count,
676 and persistence of intramammary infection in dairy cattle. J Dairy Sci. 97, 4876-4885.
677 https://doi.org/10.3168/jds.2013-7657.
678 Geoghegan, J.A., Foster, T.J., 2017. Cell Wall-Anchored Surface Proteins of Staphylococcus
679 aureus: Many Proteins, Multiple Functions. Curr Top Microbiol Immunol. 409, 95-120.
680 https://doi.org/10.1007/82_2015_5002.
681 Haley, K.P., Skaar, E.P., 2012. A battle for iron: host sequestration and Staphylococcus aureus
682 acquisition. Microbes Infect. 14, 217-227. https://doi.org/10.1016/j.micinf.2011.11.001.
683 Haveri, M., Roslöf, A., Rantala, L., Pyörälä, S., 2007. Virulence genes of bovine Staphylococcus
684 aureus from persistent and nonpersistent intramammary infections with different clinical
685 characteristics. J Appl Microbiol. 103, 993-1000. https://doi.org/10.1111/j.1365-
686 2672.2007.03356.x.

687 Hayakawa, Y., Hashimoto, N., Imaizumi, K., Kaidoh, T., Takeuchi, S., 2001. Genetic analysis of
688 exfoliative toxin A-producing Staphylococcus aureus isolated from mastitic cow's milk.
689 Vet Microbiol. 78, 39-48. https://doi.org/10.1016/s0378-1135(00)00293-5.
690 Hongo, I., Baba, T., Oishi, K., Morimoto, Y., Ito, T., Hiramatsu, K., 2009. Phenol-soluble
691 modulin alpha 3 enhances the human neutrophil lysis mediated by Panton-Valentine
692 leukocidin. J Infect Dis. 200, 715-723. https://doi.org/10.1086/605332.
693 Joensen, K.G., Scheutz, F., Lund, O., Hasman, H., Kaas, R.S., Nielsen, E.M., Aarestrup, F.M.,
694 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak
695 detection of verotoxigenic Escherichia coli. J Clin Microbiol. 52, 1501-1510.
696 https://doi.org/10.1128/jcm.03617-13.
697 Kadlec, K., Fessler, A.T., Hauschild, T., Schwarz, S., 2012. Novel and uncommon antimicrobial
698 resistance genes in livestock-associated methicillin-resistant Staphylococcus aureus. Clin
699 Microbiol Infect. 18, 745-755. https://doi.org/10.1111/j.1469-0691.2012.03842.x.
700 Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H., Phillippy, A.M., 2017. Canu:
701 scalable and accurate long-read assembly via adaptive k-mer weighting and repeat
702 separation. Genome Res. 27, 722-736. https://doi.org/10.1101/gr.215087.116.
703 Kuipers, A., Stapels, D.A.C., Weerwind, L.T., Ko, Y.P., Ruyken, M., Lee, J.C., van Kessel,
704 K.P.M., Rooijakkers, S.H.M., 2016. The Staphylococcus aureus polysaccharide capsule
705 and Efb-dependent fibrinogen shield act in concert to protect against phagocytosis.
706 Microbiology (Reading). 162, 1185-1194. https://doi.org/10.1099/mic.0.000293.
707 Kwak, Y.G., Truong-Bolduc, Q.C., Bin Kim, H., Song, K.H., Kim, E.S., Hooper, D.C., 2013.
708 Association of norB overexpression and fluoroquinolone resistance in clinical isolates of
709 Staphylococcus aureus from Korea. J Antimicrob Chemother. 68, 2766-2772.
710 https://doi.org/10.1093/jac/dkt286.
711 Lee, J.Y.H., Monk, I.R., Gonçalves da Silva, A., Seemann, T., Chua, K.Y.L., Kearns, A., Hill,
712 R., Woodford, N., Bartels, M.D., Strommenger, B., Laurent, F., Dodémont, M., Deplano,
713 A., Patel, R., Larsen, A.R., Korman, T.M., Stinear, T.P., Howden, B.P., 2018. Global
714 spread of three multidrug-resistant lineages of Staphylococcus epidermidis. Nat
715 Microbiol. 3, 1175-1185. https://doi.org/10.1038/s41564-018-0230-7.
716 Letunic, I., Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new
717 developments. Nucleic Acids Res. 47, W256-w259. https://doi.org/10.1093/nar/gkz239.
718 Li, L., Feng, W., Zhang, Z., Xue, H., Zhao, X., 2015. Macrolide-lincosamide-streptogramin
719 resistance phenotypes and genotypes of coagulase-positive Staphylococcus aureus and
720 coagulase-negative staphylococcal isolates from bovine mastitis. BMC Vet Res. 11, 168.
721 https://doi.org/10.1186/s12917-015-0492-8.
722 Lina, G., Quaglia, A., Reverdy, M.E., Leclercq, R., Vandenesch, F., Etienne, J., 1999.
723 Distribution of genes encoding resistance to macrolides, lincosamides, and
724 streptogramins among staphylococci. Antimicrob Agents Chemother. 43, 1062-1066.
725 https://doi.org/10.1128/aac.43.5.1062.
726 Lüthje, P., Schwarz, S., 2006. Antimicrobial resistance of coagulase-negative staphylococci from
727 bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance
728 phenotypes and genotypes. J Antimicrob Chemother. 57, 966-969.
729 https://doi.org/10.1093/jac/dkl061.
730 Lüthje, P., von Köckritz-Blickwede, M., Schwarz, S., 2007. Identification and characterization of
731 nine novel types of small staphylococcal plasmids carrying the lincosamide

nucleotidyltransferase gene lnu(A). J Antimicrob Chemother. 59, 600-606. https://doi.org/10.1093/jac/dkm008.
MacFadyen, A.C., Leroy, S., Harrison, E.M., Parkhill, J., Holmes, M.A., Paterson, G.K., 2019. Staphylococcus pseudoxylosus sp. nov., isolated from bovine mastitis. Int J Syst Evol Microbiol. 69, 2208-2213. https://doi.org/10.1099/ijsem.0.003416.
Madhaiyan, M., Wirth, J.S., Saravanan, V.S., 2020. Phylogenomic analyses of the Staphylococcaceae family suggest the reclassification of five species within the genus Staphylococcus as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five Staphylococcus species to Mammaliicoccus gen. nov., and the formal assignment of Nosocomiicoccus to the family Staphylococcaceae. Int J Syst Evol Microbiol. 70, 5926-5936. https://doi.org/10.1099/ijsem.0.004498.
Mahato, S., Mistry, H.U., Chakraborty, S., Sharma, P., Saravanan, R., Bhandari, V., 2017. Identification of Variable Traits among the Methicillin Resistant and Sensitive Coagulase Negative Staphylococci in Milk Samples from Mastitic Cows in India. Front Microbiol. 8, 1446. https://doi.org/10.3389/fmicb.2017.01446.
Malachowa, N., DeLeo, F.R., 2010. Mobile genetic elements of Staphylococcus aureus. Cell Mol Life Sci. 67, 3057-3071. https://doi.org/10.1007/s00018-010-0389-4.
Małyszko, I., Schwarz, S., Hauschild, T., 2014. Detection of a new mecC allotype, mecC2, in methicillin-resistant Staphylococcus saprophyticus. J Antimicrob Chemother. 69, 2003-2005. https://doi.org/10.1093/jac/dku043.
Melchior, M.B., van Osch, M.H., Graat, R.M., van Duijkeren, E., Mevius, D.J., Nielen, M., Gaastra, W., Fink-Gremmels, J., 2009. Biofilm formation and genotyping of Staphylococcus aureus bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. Vet Microbiol. 137, 83-89. https://doi.org/10.1016/j.vetmic.2008.12.004.
Melchior, M.B., van Osch, M.H., Lam, T.J., Vernooij, J.C., Gaastra, W., Fink-Gremmels, J., 2011. Extended biofilm susceptibility assay for Staphylococcus aureus bovine mastitis isolates: evidence for association between genetic makeup and biofilm susceptibility. J Dairy Sci. 94, 5926-5937. https://doi.org/10.3168/jds.2011-4243.
Mello, P.L., Moraes Riboli, D.F., Pinheiro, L., de Almeida Martins, L., Vasconcelos Paiva Brito, M.A., Ribeiro de Souza da Cunha Mde, L., 2016. Detection of Enterotoxigenic Potential and Determination of Clonal Profile in Staphylococcus aureus and Coagulase-Negative Staphylococci Isolated from Bovine Subclinical Mastitis in Different Brazilian States. Toxins (Basel). 8, 104. https://doi.org/10.3390/toxins8040104.
Miragaia, M., 2018. Factors Contributing to the Evolution of mecA-Mediated β-lactam Resistance in Staphylococci: Update and New Insights From Whole Genome Sequencing (WGS). Front Microbiol. 9, 2723. https://doi.org/10.3389/fmicb.2018.02723.
Monecke, S., Engelmann, I., Archambault, M., Coleman, D.C., Coombs, G.W., Cortez de Jäckel, S., Pelletier-Jacques, G., Schwarz, S., Shore, A.C., Slickers, P., Ehricht, R., 2012. Distribution of SCCmec-associated phenol-soluble modulin in staphylococci. Mol Cell Probes. 26, 99-103. https://doi.org/10.1016/j.mcp.2012.01.001.
Møretrø, T., Hermansen, L., Holck, A.L., Sidhu, M.S., Rudi, K., Langsrud, S., 2003. Biofilm formation and the presence of the intercellular adhesion locus ica among staphylococci from food and food processing environments. Appl Environ Microbiol. 69, 5648-5655. https://doi.org/10.1128/aem.69.9.5648-5655.2003.

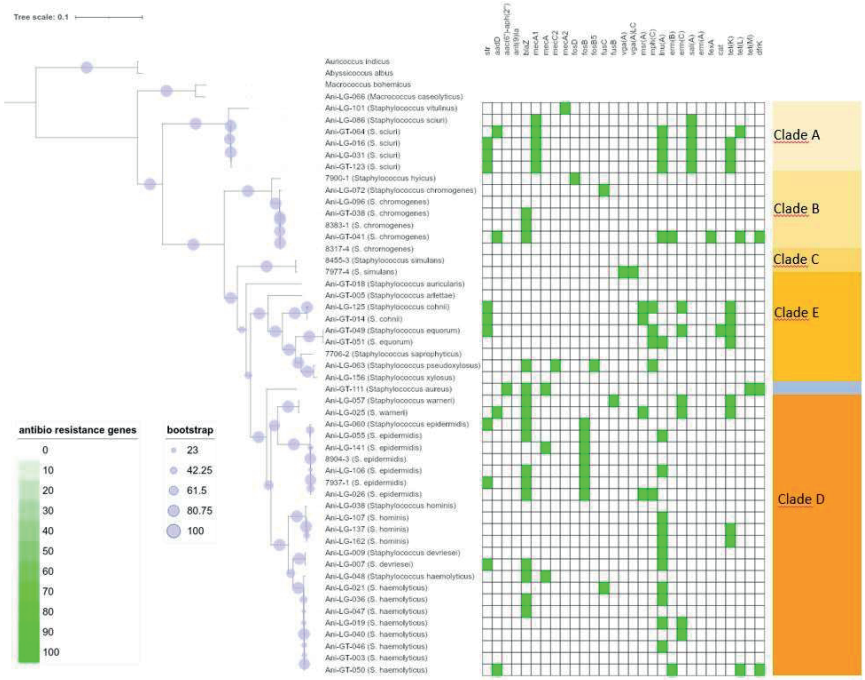
778 Naushad, S., Barkema, H.W., Luby, C., Condas, L.A., Nobrega, D.B., Carson, D.A., De Buck, J.,
779 2016. Comprehensive Phylogenetic Analysis of Bovine Non-aureus Staphylococci
780 Species Based on Whole-Genome Sequencing. Front Microbiol. 7, 1990.
781 https://doi.org/10.3389/fmicb.2016.01990.
782 Naushad, S., Naqvi, S.A., Nobrega, D., Luby, C., Kastelic, J.P., Barkema, H.W., De Buck, J.,
783 2019. Comprehensive Virulence Gene Profiling of Bovine Non-aureus Staphylococci
784 Based on Whole-Genome Sequencing Data. mSystems.
785 https://doi.org/10.1128/mSystems.00098-18.
786 Nia, Y., Mutel, I., Assere, A., Lombard, B., Auvray, F., Hennekinne, J.A., 2016. Review Over a
787 3-Year Period of European Union Proficiency Tests for Detection of Staphylococcal
788 Enterotoxins in Food Matrices. Toxins (Basel). 8, 107.
789 https://doi.org/10.3390/toxins8040107.
790 Nobrega, D.B., Naushad, S., Naqvi, S.A., Condas, L.A.Z., Saini, V., Kastelic, J.P., Luby, C., De
791 Buck, J., Barkema, H.W., 2018. Prevalence and Genetic Basis of Antimicrobial
792 Resistance in Non-aureus Staphylococci Isolated from Canadian Dairy Herds. Front
793 Microbiol. 9, 256. https://doi.org/10.3389/fmicb.2018.00256.
794 Olde Riekerink, R.G., Barkema, H.W., Kelton, D.F., Scholl, D.T., 2008. Incidence rate of
795 clinical mastitis on Canadian dairy farms. J Dairy Sci. 91, 1366-1377.
796 https://doi.org/10.3168/jds.2007-0757.
797 Olsen, J.E., Christensen, H., Aarestrup, F.M., 2006. Diversity and evolution of blaZ from
798 Staphylococcus aureus and coagulase-negative staphylococci. J Antimicrob Chemother.
799 57, 450-460. https://doi.org/10.1093/jac/dki492.
800 Osterås, O., Sølverød, L., Reksen, O., 2006. Milk culture results in a large Norwegian survey--
801 effects of season, parity, days in milk, resistance, and clustering. J Dairy Sci. 89, 1010-
802 1023. https://doi.org/10.3168/jds.S0022-0302(06)72167-1.
803 Otto, M., 2013. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA
804 infection: Staphylococcal commensal species such as Staphylococcus epidermidis are
805 being recognized as important sources of genes promoting MRSA colonization and
806 virulence. Bioessays. 35, 4-11. https://doi.org/10.1002/bies.201200112.
807 Otto, M., 2014. Phenol-soluble modulins. Int J Med Microbiol. 304, 164-169.
808 https://doi.org/10.1016/j.ijmm.2013.11.019.
809 Piddock, L.J., 2006. Clinically relevant chromosomally encoded multidrug resistance efflux
810 pumps in bacteria. Clin Microbiol Rev. 19, 382-402.
811 https://doi.org/10.1128/cmr.19.2.382-402.2006.
812 Piessens, V., De Vliegher, S., Verbist, B., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx,
813 M., Van Coillie, E., 2012. Characterization of coagulase-negative staphylococcus species
814 from cows' milk and environment based on bap, icaA, and mecA genes and phenotypic
815 susceptibility to antimicrobials and teat dips. J Dairy Sci. 95, 7027-7038.
816 https://doi.org/10.3168/jds.2012-5400.
817 Piessens, V., Van Coillie, E., Verbist, B., Supré, K., Braem, G., Van Nuffel, A., De Vuyst, L.,
818 Heyndrickx, M., De Vliegher, S., 2011. Distribution of coagulase-negative
819 Staphylococcus species from milk and environment of dairy cows differs between herds.
820 J Dairy Sci. 94, 2933-2944. https://doi.org/10.3168/jds.2010-3956.
821 Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., Honkanen-Buzalski, T., 2004. Bovine mastitis in
822 Finland 2001--prevalence, distribution of bacteria, and antimicrobial resistance. J Dairy
823 Sci. 87, 2433-2441. https://doi.org/10.3168/jds.S0022-0302(04)73366-4.

824 Poole, K., 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann Med. 39, 162-176.
825 https://doi.org/10.1080/07853890701195262.
826 Pyörälä, S., Taponen, S., 2009. Coagulase-negative staphylococci-emerging mastitis pathogens.
827 Vet Microbiol. 134, 3-8. https://doi.org/10.1016/j.vetmic.2008.09.015.
828 Qin, L., McCausland, J.W., Cheung, G.Y., Otto, M., 2016. PSM-Mec-A Virulence Determinant
829 that Connects Transcriptional Regulation, Virulence, and Antibiotic Resistance in
830 Staphylococci. Front Microbiol. 7, 1293. https://doi.org/10.3389/fmicb.2016.01293.
831 Reksen, O., Sølverød, L., Branscum, A.J., Osterås, O., 2006. Relationships between milk culture
832 results and treatment for clinical mastitis or culling in Norwegian dairy cattle. J Dairy
833 Sci. 89, 2928-2937. https://doi.org/10.3168/jds.S0022-0302(06)72565-6.
834 Salimena, A.P., Lange, C.C., Camussone, C., Signorini, M., Calvinho, L.F., Brito, M.A., Borges,
835 C.A., Guimarães, A.S., Ribeiro, J.B., Mendonça, L.C., Piccoli, R.H., 2016. Genotypic
836 and phenotypic detection of capsular polysaccharide and biofilm formation in
837 Staphylococcus aureus isolated from bovine milk collected from Brazilian dairy farms.
838 Vet Res Commun. 40, 97-106. https://doi.org/10.1007/s11259-016-9658-5.
839 Santos Costa, S., Viveiros, M., Rosato, A.E., Melo-Cristino, J., Couto, I., 2015. Impact of efflux
840 in the development of multidrug resistance phenotypes in Staphylococcus aureus. BMC
841 Microbiol. 15, 232. https://doi.org/10.1186/s12866-015-0572-8.
842 Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 30, 2068-
843 2069. https://doi.org/10.1093/bioinformatics/btu153.
844 Seemann, T., Edwards, R., da Silva, A.G., Kiil, K. 2020. Shovill.
845 Sheldon, J.R., Heinrichs, D.E., 2015. Recent developments in understanding the iron acquisition
846 strategies of gram positive pathogens. FEMS Microbiol Rev. 39, 592-630.
847 https://doi.org/10.1093/femsre/fuv009.
848 Spaulding, A.R., Salgado-Pabón, W., Kohler, P.L., Horswill, A.R., Leung, D.Y., Schlievert,
849 P.M., 2013. Staphylococcal and streptococcal superantigen exotoxins. Clin Microbiol
850 Rev. 26, 422-447. https://doi.org/10.1128/cmr.00104-12.
851 Supré, K., Haesebrouck, F., Zadoks, R.N., Vaneechoutte, M., Piepers, S., De Vliegher, S., 2011.
852 Some coagulase-negative Staphylococcus species affect udder health more than others. J
853 Dairy Sci. 94, 2329-2340. https://doi.org/10.3168/jds.2010-3741.
854 Tam, K., Torres, V.J., 2019. Staphylococcus aureus Secreted Toxins and Extracellular Enzymes.
855 Microbiol Spectr. 7https://doi.org/10.1128/microbiolspec.GPP3-0039-2018.
856 Taponen, S., Björkroth, J., Pyörälä, S., 2008. Coagulase-negative staphylococci isolated from
857 bovine extramammary sites and intramammary infections in a single dairy herd. J Dairy
858 Res. 75, 422-429. https://doi.org/10.1017/s0022029908003312.
859 Taponen, S., Liski, E., Heikkilä, A.M., Pyörälä, S., 2017. Factors associated with intramammary
860 infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus
861 aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or
862 Escherichia coli. J Dairy Sci. 100, 493-503. https://doi.org/10.3168/jds.2016-11465.
863 Thompson, T., Brown, P.D., 2014. Comparison of antibiotic resistance, virulence gene profiles,
864 and pathogenicity of methicillin-resistant and methicillin-susceptible Staphylococcus
865 aureus using a Caenorhabditis elegans infection model. Pathog Glob Health. 108, 283-
866 291. https://doi.org/10.1179/2047773214y.0000000155.
867 Tremblay, Y.D., Lamarche, D., Chever, P., Haine, D., Messier, S., Jacques, M., 2013.
868 Characterization of the ability of coagulase-negative staphylococci isolated from the milk

of Canadian farms to form biofilms. J Dairy Sci. 96, 234-246. https://doi.org/10.3168/jds.2012-5795.
Valckenier, D., Piepers, S., De Visscher, A., Bruckmaier, R.M., De Vliegher, S., 2019. Effect of intramammary infection with non-aureus staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. J Dairy Sci. 102, 6442-6453. https://doi.org/10.3168/jds.2018-15913.
Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F., De Vliegher, S., 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Vet J. 203, 44-51. https://doi.org/10.1016/j.tvjl.2014.11.001.
Vaughn, J.M., Abdi, R.D., Gillespie, B.E., Kerro Dego, O., 2020. Genetic diversity and virulence characteristics of Staphylococcus aureus isolates from cases of bovine mastitis. Microb Pathog. 144, 104171. https://doi.org/10.1016/j.micpath.2020.104171.
Verkaik, N.J., Benard, M., Boelens, H.A., de Vogel, C.P., Nouwen, J.L., Verbrugh, H.A., Melles, D.C., van Belkum, A., van Wamel, W.J., 2011. Immune evasion cluster-positive bacteriophages are highly prevalent among human Staphylococcus aureus strains, but they are not essential in the first stages of nasal colonization. Clin Microbiol Infect. 17, 343-348. https://doi.org/10.1111/j.1469-0691.2010.03227.x.
Vitali, L.A., Petrelli, D., Lamikanra, A., Prenna, M., Akinkunmi, E.O., 2014. Diversity of antibiotic resistance genes and staphylococcal cassette chromosome mec elements in faecal isolates of coagulase-negative staphylococci from Nigeria. BMC Microbiol. 14, 106. https://doi.org/10.1186/1471-2180-14-106.
Vrieling, M., Boerhout, E.M., van Wigcheren, G.F., Koymans, K.J., Mols-Vorstermans, T.G., de Haas, C.J., Aerts, P.C., Daemen, I.J., van Kessel, K.P., Koets, A.P., Rutten, V.P., Nuijten, P.J., van Strijp, J.A., Benedictus, L., 2016. LukMF' is the major secreted leukocidin of bovine Staphylococcus aureus and is produced in vivo during bovine mastitis. Sci Rep. 6, 37759. https://doi.org/10.1038/srep37759.
Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C.A., Zeng, Q., Wortman, J., Young, S.K., Earl, A.M., 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One. 9, e112963. https://doi.org/10.1371/journal.pone.0112963.
Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., DeLeo, F.R., Otto, M., 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med. 13, 1510-1514. https://doi.org/10.1038/nm1656.
Wassenaar, T.M., Ussery, D.W., Ingmer, H., 2016. The qacC Gene Has Recently Spread between Rolling Circle Plasmids of Staphylococcus, Indicative of a Novel Gene Transfer Mechanism. Front Microbiol. 7, 1528. https://doi.org/10.3389/fmicb.2016.01528.
Wu, S., Piscitelli, C., de Lencastre, H., Tomasz, A., 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of mecA from a methicillin susceptible strain of Staphylococcus sciuri. Microb Drug Resist. 2, 435-441. https://doi.org/10.1089/mdr.1996.2.435.
Wuytack, A., De Visscher, A., Piepers, S., Boyen, F., Haesebrouck, F., De Vliegher, S., 2020. Distribution of non-aureus staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential. J Dairy Sci. 103, 10658-10675. https://doi.org/10.3168/jds.2020-18265.

915 Xu, J., Tan, X., Zhang, X., Xia, X., Sun, H., 2015. The diversities of staphylococcal species,
916 virulence and antibiotic resistance genes in the subclinical mastitis milk from a single
917 Chinese cow herd. Microb Pathog. 88, 29-38.
918 https://doi.org/10.1016/j.micpath.2015.08.004.
919 Yamada, T., Tochimaru, N., Nakasuji, S., Hata, E., Kobayashi, H., Eguchi, M., Kaneko, J.,
920 Kamio, Y., Kaidoh, T., Takeuchi, S., 2005. Leukotoxin family genes in Staphylococcus
921 aureus isolated from domestic animals and prevalence of lukM-lukF-PV genes by
922 bacteriophages in bovine isolates. Vet Microbiol. 110, 97-103.
923 https://doi.org/10.1016/j.vetmic.2005.07.006.
924 Zankari, E., Allesøe, R., Joensen, K.G., Cavaco, L.M., Lund, O., Aarestrup, F.M., 2017.
925 PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance
926 associated with chromosomal point mutations in bacterial pathogens. J Antimicrob
927 Chemother. 72, 2764-2768. https://doi.org/10.1093/jac/dkx217.
928 Zhang, H.Z., Hackbarth, C.J., Chansky, K.M., Chambers, H.F., 2001. A proteolytic
929 transmembrane signaling pathway and resistance to beta-lactams in staphylococci.
930 Science. 291, 1962-1965. https://doi.org/10.1126/science.1055144.
931 Zhou, Y., Antignac, A., Wu, S.W., Tomasz, A., 2008. Penicillin-binding proteins and cell wall
932 composition in beta-lactam-sensitive and -resistant strains of Staphylococcus sciuri. J
933 Bacteriol. 190, 508-514. https://doi.org/10.1128/jb.01549-07.
934 Åvall-Jääskeläinen, S., Taponen, S., Kant, R., Paulin, L., Blom, J., Palva, A., Koort, J., 2018.
935 Comparative genome analysis of 24 bovine-associated Staphylococcus isolates with
936 special focus on the putative virulence genes. PeerJ. 6, e4560.
937 https://doi.org/10.7717/peerj.4560.

938



939

940 Figure 1. Phylogeny and antimicrobial resistance genes of non-*aureus* staphylococci

941

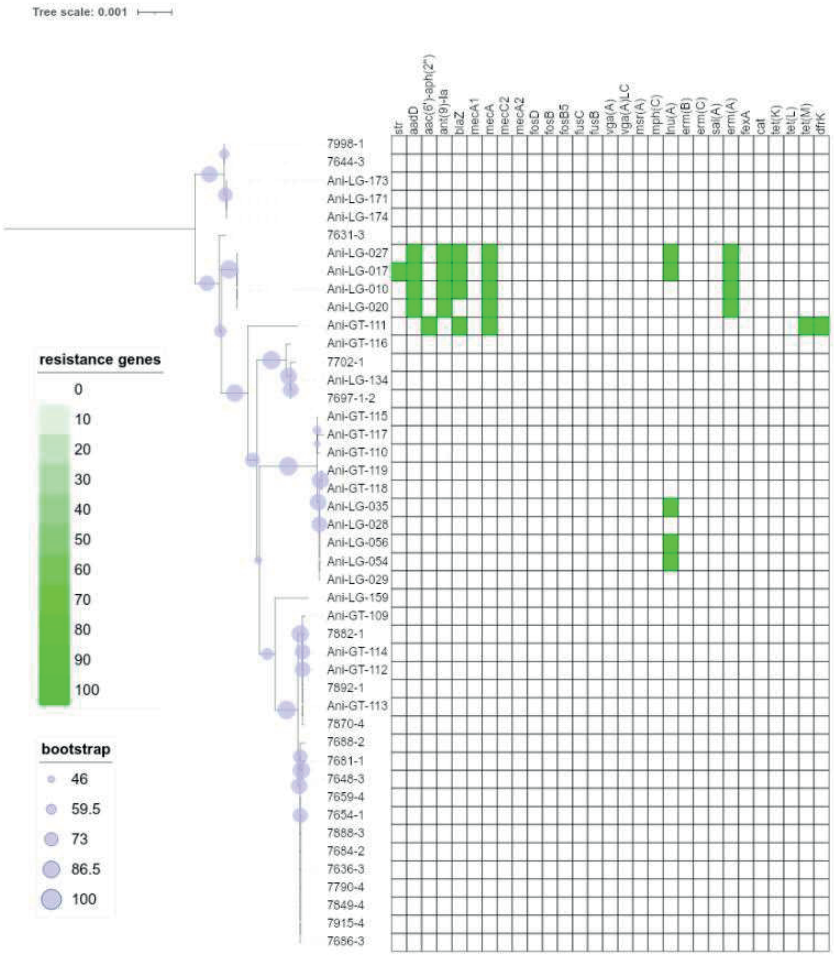


Figure 2. Phylogeny and antimicrobial resistance genes of *Staphylococcus aureus*

945

946 Figure 3. Phylogeny of lincosamide resistance gene *lnuA*

947

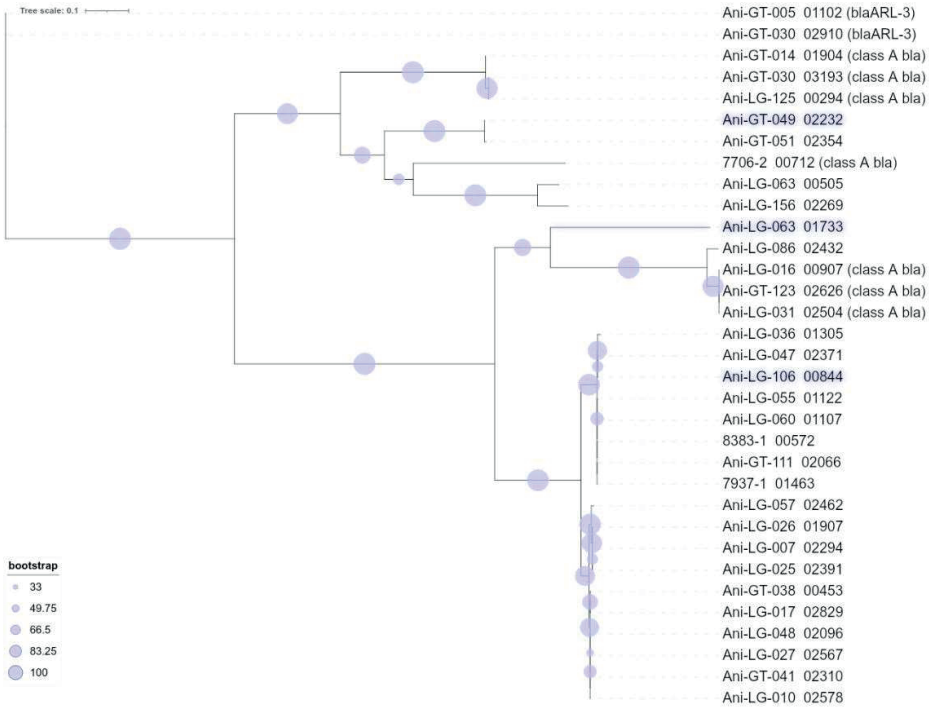


Figure 4. Phylogeny of penicillin resistance gene *blaZ*

Table 1. Distribution of virulence genes involved in adherence in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

Virulence factors	Related gene	SAU[1] (45)	SAU[1] (%)	NAS (44)	NAS (%)	Clade A		Clade B		Clade C	Clade D					Clade E					
						SVI[1] (1)	SSC[1] (3)	SHY[1] (1)	SCH[1] (8)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (9)	SWA[1] (2)	SEP (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SEQ[1] (2)	SSA[1] (1)	SXY[1] (2)
Accumulation associated protein	*aap*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Biofilm-associated surface protein Bap	*bap*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Autolysin	*atl*	45	100%	21	44%	1	0	1	1	0	0	0	9	2	7	0	0	0	0	0	0
Clumping factor A	*clfA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clumping factor B	*clfB*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Collagen adhesin	*cna*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Elastin binding protein	*ebp*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell wall associated fibronectin binding protein	*fnbA*	31	69%	5	10%	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0
	fnbB	41	91%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fibronectin binding protein	*fnbA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	fnbB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Extracellular adherence protein/MHC analogous protein	*eap/ map*	7	16%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell wall surface anchor family protein	*sasC*	26	58%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sasG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sasP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Intracellular adhesin (*ica* operon)	*icaA*	41	91%	6	12%	0	3	0	0	0	0	0	0	0	0	0	0	2	0	0	1
	icaB	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	icaC	45	100%	3	6%	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1
	icaD	45	100%	2	4%	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
	icaR	45	100%	1	2%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Ser-Asp rich fibrinogen-binding proteins	*sdrC*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrH	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sdrI	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*, SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 2. Distribution of exoenzyme virulence genes in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

						Clade A		Clade B		Clade C	Clade D					Clade E					
Virulence factors	Related gene	SAU[1] (45)	SAU[1] (%)	NAS (48)	NAS (%)	SVI[1] (1)	SSC[1] (5)	SHY[1] (1)	SCH[1] (6)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (9)	SWA[1] (2)	SEP[1] (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SEQ[1] (2)	SSA[1] (1)	SXY[1] (2)
Adenosine synthase A	*adsA*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aureolysin	*aur*	43	96%	27	56%	1	5	1	6	0	1	0	0	2	7	0	0	0	2	1	1
Cysteine protease (Staphopain)	*sspA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sspB	45	100%	12	25%	0	0	1	0	2	0	0	0	2	7	0	0	0	0	0	0
	sspC	45	100%	2	4%	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
	sspD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sspE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sspF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hyaluronat lyase	*hysA*	44	98%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Lipase	*lip*	45	100%	11	23%	0	0	0	0	2	0	0	0	0	7	0	0	2	0	0	0
	geh	45	100%	24	50%	0	0	0	0	2	0	0	8	2	7	0	0	2	0	1	1
Serine protease	*splA*	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	splB	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	splC	43	96%	2	4%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	splD	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	splE	43	96%	5	10%	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	splF	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coagulase	*coa*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylokinase	*sak*	2	4%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thermonuclease	*nuc*	45	100%	36	75%	0	0	1	0	2	4	2	9	2	7	1	1	2	2	1	2
von Willebrand factor binding protein	*vWbp*	42	93%	5	10%	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*, SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 3. Distribution of virulence genes involved in host immune evasion in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

						Clade A		Clade B		Clade C	Clade D					Clade E					
Virulence factors	Related gene	SAU[1] (45)	SAU[1] %	NAS (48)	NAS %	SVI[1] (1)	SSC[1] (5)	SHY[1] (1)	SCH[1] (6)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (9)	SWA[1] (2)	SEP[1] (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SEQ[1] (2)	SSA[1] (1)	SXY[1] (2)
Capsule synthesis	*capA*	45	100%	19	40%	1	0	1	1	2	3	2	8	0	0	0	1	0	0	0	0
	capB	45	100%	19	40%	1	0	1	1	2	3	2	8	0	0	0	1	0	0	0	0
	capC	45	100%	21	44%	1	5	1	1	2	3	2	8	2	0	0	1	0	0	0	0
	capD	39	87%	18	37%	1	0	1	0	2	3	2	8	0	0	0	1	0	0	0	0
	capE	45	100%	9	19%	1	0	1	1	0	0	1	4	0	0	0	1	0	0	0	0
	capF	45	100%	8	17%	1	0	1	1	0	0	0	4	0	0	0	1	0	0	0	0
	capG	45	100%	8	17%	1	0	1	1	0	0	0	4	0	0	0	1	0	0	0	0
	capH	45	100%	5	10%	0	0	1	1	0	0	0	2	0	0	0	1	0	0	0	0
	capI	45	100%	6	12%	0	0	0	1	0	0	0	4	0	0	0	1	0	0	0	0
	capJ	45	100%	5	10%	0	0	1	0	0	0	0	4	0	0	0	0	0	0	0	0
	capK	45	100%	6	12%	0	0	1	1	0	0	0	4	0	0	0	0	0	0	0	0
	capL	45	100%	6	12%	1	0	1	1	0	0	0	2	0	0	0	1	0	0	0	0
	capM	45	100%	19	40%	1	0	1	1	2	3	2	8	0	0	0	1	0	0	0	0
	capN	45	100%	4	8%	1	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0
	capO	45	100%	19	40%	1	5	1	6	0	0	0	5	0	0	0	1	0	0	0	0
	capP	45	100%	48	100%	1	5	1	6	2	4	2	9	2	7	1	1	2	2	1	1
Chemotaxsis inhibitory protein	*chp*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcal complement inhibitor	*scn*	44	98%	5	10%	0	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcal protein A	*spa*	34	76%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcal binder of immunoglobulin	*sbi*	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*, SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 4. Distribution of virulence genes involved in iron uptake and metabolism in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

Virulence factors	Related gene	SAU[1] (45)	SAU[1] %	NAS (48)	NAS %	Clade A		Clade B		Clade C	Clade D					Clade E					
						SVI[1] (1)	SSC[1] (5)	SHY[1] (1)	SCH[1] (6)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (9)	SWA[1] (2)	SEP[1] (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SEQ[1] (2)	SSA[1] (1)	SXY[1] (2)
Iron-regulated surface determinant genes	*isdA*	44	98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdB	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdC	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdD	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdE	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdF	45	100%	36	75%	1	5	1	0	2	0	2	8	2	7	1	1	2	2	0	2
	isdG	45	100%	30	60%	0	0	1	6	2	4	2	0	2	7	1	0	0	2	1	2
	isdH	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	isdI	45	100%	34	71%	1	0	1	6	2	4	2	0	2	6	1	1	2	2	1	2
ABC transporter (siderophore receptor)	*htsA*	45	100%	42	88%	1	2	1	6	2	1	2	9	2	7	1	1	2	2	1	2
	htsB	45	100%	46	96%	1	2	1	6	2	4	2	9	2	7	1	1	2	2	1	2
	htsC	45	100%	48	100%	1	5	1	6	2	4	2	9	2	7	1	1	2	2	1	2
	sfaA	45	100%	35	73%	0	0	1	6	2	0	2	9	2	7	1	0	2	0	1	2
	sfaB	45	100%	42	88%	0	0	1	6	2	4	2	9	2	7	1	1	2	2	1	2
	sfaC	45	100%	42	88%	0	0	1	6	2	4	2	9	2	7	1	1	2	2	1	2
	sfaD	45	100%	41	85%	0	0	1	6	2	3	2	9	2	7	1	1	2	2	1	2
Staphyloferrin A and B synthesis-related genes	*sirA*	45	100%	31	65%	1	2	1	6	0	0	2	9	2	0	1	1	2	2	0	2
	sirB	45	100%	40	83%	0	2	1	6	2	0	2	9	2	7	1	1	2	2	1	2
	sirC	45	100%	26	54%	1	0	1	5	2	0	2	9	2	0	0	1	0	2	1	0
	sbnA	45	100%	48	100%	1	5	1	6	2	4	2	9	2	7	1	1	2	2	1	2
	sbnB	45	100%	6	12%	0	0	1	1	0	0	0	0	0	0	0	1	2	2	0	0
	sbnC	45	100%	4	8%	0	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnD	45	100%	4	8%	0	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnE	44	100%	4	8%	0	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnF	45	100%	4	8%	0	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnG	45	100%	4	8%	0	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnH	45	100%	5	10%	1	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0
	sbnI	45	100%	3	6%	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
Sortase B	*srtB*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*,

SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 5. Distribution of toxin, type IIV secretion and phenol-soluble modulin genes in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

Virulence factors	Related gene	SAU (45)	SAU %	NAS (48)	NAS %	Clade A		Clade B		Clade C	Clade D					Clade E					
						SVI (1)	SSC (5)	SHY (1)	SCH (6)	SSI (2)	SHO (4)	SDE (2)	SHA (9)	SWA (2)	SEP (7)	SAC (11)	SAR (1)	SCO (2)	SEQ (2)	SSA (1)	SXY (2)
Alpha hemolysin	*hla*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta hemolysin	*hlb*	0	0	7	15%	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0
Delta hemolysin	*hld*	11	24%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gamma hemolysin	*hlg1*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	hlgB	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	hlgC	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leukocidin M	*lukM*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	lukF-like	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Panton-Valentin leukocidin	*lukS-PV*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	lukF-PV	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leukotoxin D	*lukD*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leukotoxin E	*lukE*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxic shock syndrome toxin	*tst*	13	29%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exfoliative toxin type A	*eta*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exfoliative toxin type B	*etb*	0	0	5	10%	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exfoliative toxin type C	*etc*	44	98%	48	100%	1	5	1	6	2	4	2	9	2	7	1	2	1	2	1	2
Exfoliative toxin type D	*etd*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Type VII secretion system	*esxA*	37	82%	3	6%	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0
	esxB	45	100%	3	6%	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0
	esxC	44	98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	esxA	36	80%	3	6%	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0
	esxB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	esxC	43	96%	3	6%	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0
	esxA	45	100%	3	6%	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0
	esxB	44	98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenol soluble modulins alpha	*PSMα1*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PSMα2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PSMα3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PSMα4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PSMαnov	0	0	6	12%	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenol soluble modulins beta	*PSMβ1*	45	100%	41	85%	0	0	1	6	2	4	2	9	2	7	1	0	2	2	1	2
	PSMβ2	45	100%	41	85%	0	0	1	6	2	4	2	9	2	7	1	0	2	2	1	2
	PSMβ3	45	100%	37	77%	0	0	0	6	0	4	2	9	2	7	1	0	2	1	1	2
	PSMβ4	45	100%	39	81%	0	0	1	6	0	4	2	9	2	7	1	0	2	2	1	2
	PSMβ5	45	100%	39	81%	0	0	1	6	0	4	2	9	2	7	1	0	2	2	1	2
	PSMβ6	45	100%	39	81%	0	0	1	6	0	4	2	9	2	7	1	0	2	2	1	2

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*,

SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*,
SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 6. Distribution of exotoxin genes in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

						Clade A		Clade B		Clade C	Clade D					Clade E					
Virulence factors	Related gene	SAU[1] (43)	SAU[1] %	NAS (48)	NAS %	SVI[1] (1)	SSC[1] (2)	SHY[1] (3)	SCH[1] (6)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (8)	SWA[1] (2)	SEP[1] (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SHQ[1] (2)	SHA[1] (1)	SXC[1] (2)
Staph exotoxin 1	*set1*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 2	*set2*	1	2%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 3	*set3*	45	100%	5	10%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 4	*set4*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 5	*set5*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 6	*set6*	45	100%	5	10%	0	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 7	*set7*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 8	*set8*	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 9	*set9*	29	64%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 10	*set10*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 11	*set11*	45	100%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 12	*set12*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 13	*set13*	45	100%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 15	*set15*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 16	*set16*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 17	*set17*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 18	*set18*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 19	*set19*	23	51%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 20	*set20*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 21	*set21*	45	100%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 22	*set22*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 23	*set23*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 24	*set24*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 25	*set25*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 26	*set26*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 30	*set30*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 31	*set31*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 32	*set32*	43	96%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 33	*set33*	32	71%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 34	*set34*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 35	*set35*	45	100%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 36	*set36*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 37	*set37*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 38	*set38*	33	73%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 39	*set39*	45	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph exotoxin 40	*set40*	45	100%	6	12%	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*,

SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table 7. Distribution of enterotoxin genes in all species. NAS are shown according to clade. Green color indicates no virulence genes genes, light orange indicates the presence of virulence genes. For columns with *S. aureus* and total NAS dark orange indicates virulence gene present in over 50% of isolates

						Clade A		Clade B		Clade C	Clade D					Clade E					
Virulence factors	Related gene	SAU[1] (45)	SAU[1] %	NAS (48)	NAS %	SVI[1] (1)	SSC[1] (5)	SHY[1] (1)	SCH[1] (6)	SSI[1] (2)	SHO[1] (4)	SDE[1] (2)	SHA[1] (9)	SWA[1] (2)	SEP[1] (7)	SAC[1] (1)	SAR[1] (1)	SCO[1] (2)	SEQ[1] (2)	SSA[1] (1)	SXY[1] (2)
Enterotoxin A	*sea*	28	62%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin B	*seb*	21	46%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin C	*sec*	20	44%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin D	*sed*	3	7%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin E	*see*	16	35%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin G	*seg*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin H	*she*	15	33%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin I	*sei*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin J	*sej*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like K	*selk*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like L	*sell*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like M	*selm*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like N	*seln*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like O	*selo*	23	51%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like P	*selp*	23	51%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like Q	*selq*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like R	*selr*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like U	*selu*	21	46%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin-like V	*selv*	18	40%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin Yent1	*yent1*	14	31%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterotoxin Yent2	*yent2*	45	100%	1	2%	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Species abbreviations: SAU=*S. aureus*, SVI=*S. vitulinus*, SSC=*S. sciuri*, SHY=*S. hyicus*, SCH=*S. chromogenes*, SSI=*S. simulans*, SHO=*S. hominis*, SDE=*S. devriesei*, SHA=*S. haemolyticus*, SWA=*S. warneri*, SEP=*S. epidermidis*, SAC=*S. auricularis*, SAR=*S. arlettae*, SCO=*S. cohnii*, SEQ=*S. equorum*, SSA=*S. saprophyticus*, SXY=*S. xylosus*

Table S1.

Table S1. Statistics on whole genome sequencing assemblies

ID_platform	ID	N50	Total length (> 500 nt)	Median coverage depth	Nr contigs
1	7631-3	313598	2748195	224,52	26
2	7636-3	171970	2770533	164,50	48
3	7644-3	241598	2733537	109,52	28
5	7648-3	193545	2799172	236,54	47
7	7654-1	161025	2798571	249,39	54
9	7659-4	223310	2778760	131,05	39
11	7681-1	254662	2798528	209,20	49
12	7684-2	222879	2783403	155,12	49
13	7686-3	231801	2760802	190,61	31
14	7688-2	194907	2746708	171,44	34
15	7697-1-2	583493	2711923	174,05	22
16	7702-1	294681	2712468	188,05	22
17	7706-2	124111	2611115	138,92	45
20	7790-4	209622	2786357	111,18	43
24	7849-4	160983	2796756	168,40	56
26	7870-4	125236	2697770	180,79	36
28	7882-1	55914	2742205	154,48	87
29	7888-3	255043	2781927	142,11	39
30	7892-1	125234	2743849	242,45	35
31	7900-1	355845	2409102	174,08	21
33	7915-4	226426	2744674	164,96	32
37	7937-1	166541	2433642	210,91	35
40	7977-4	571433	2678019	151,87	18
36	7998-1	173685	2724860	185,05	31
50	8317-4	249840	2293386	148,19	20
55	8383-1	409797	2311028	231,84	24
59	8455-3	255688	2641141	133,73	30
75	8904-3	193228	2479036	192,92	31
103	Ani-GT-003	203152	2446270	66,05	28
105	Ani-GT-005	214986	2578935	197,35	33
114	Ani-GT-014	77978	2617042	84,73	62
117	Ani-GT-018	302968	2191669	177,71	19
134	Ani-GT-038	595257	2310546	77,08	21
137	Ani-GT-041	594702	2346453	114,57	17
142	Ani-GT-046	185581	2491123	161,42	37
145	Ani-GT-049	467855	2751026	187,32	34
146	Ani-GT-050	248558	2451914	194,25	30
147	Ani-GT-051	589909	2675071	183,26	21
154	Ani-GT-064	70535	2635668	183,05	89
162	Ani-GT-109	238784	2743966	177,24	34
163	Ani-GT-110	250775	2661040	166,61	35
164	Ani-GT-111	149833	2782588	103,64	50
165	Ani-GT-112	119889	2740164	95,17	49
166	Ani-GT-113	132392	2739074	88,43	35

167	Ani-GT-114	163341	2698699	182,75	33
168	Ani-GT-115	117744	2731014	56,99	55
169	Ani-GT-116	122642	2745452	137,82	60
170	Ani-GT-117	191839	2690311	76,59	33
171	Ani-GT-118	251011	2662568	119,18	40
172	Ani-GT-119	250975	2650274	133,38	26
173	Ani-GT-123	69924	2728565	144,98	97
178	Ani-LG-007	568121	2345108	87,56	21
180	Ani-LG-009	559826	2300414	84,31	16
265	Ani-LG-010	190473	2827737	86,60	37
187	Ani-LG-016	79339	2651933	118,57	93
188	Ani-LG-017	156740	2939070	158,64	56
190	Ani-LG-019	203189	2491852	104,29	30
191	Ani-LG-020	306642	2798401	101,17	31
192	Ani-LG-021	148121	2523226	156,38	27
196	Ani-LG-025	320365	2575090	160,34	38
268	Ani-LG-026	133485	2391707	161,54	39
198	Ani-LG-027	315742	2876308	122,51	45
199	Ani-LG-028	251015	2705339	186,24	39
269	Ani-LG-029	288619	2704706	148,27	38
202	Ani-LG-031	69924	2656581	102,28	101
206	Ani-LG-035	250815	2709865	177,88	41
207	Ani-LG-036	538967	2421282	227,33	17
262	Ani-LG-038	488485	2189080	208,60	21
210	Ani-LG-040	253355	2539576	161,62	39
216	Ani-LG-047	108713	2450895	155,05	59
217	Ani-LG-048	101114	2347821	176,76	43
222	Ani-LG-054	250889	2710055	158,73	41
223	Ani-LG-055	110717	2529786	223,51	50
224	Ani-LG-056	288619	2709738	172,75	40
225	Ani-LG-057	318516	2553959	273,55	26
227	Ani-LG-060	119549	2491938	248,91	47
228	Ani-LG-063	281839	3130640	121,35	31
231	Ani-LG-066	217314	2393814	195,57	37
237	Ani-LG-072	293655	2307564	156,15	23
249	Ani-LG-086	82388	2601163	158,66	81
254	Ani-LG-096	249419	2368674	165,55	19
258	Ani-LG-101	289393	2482705	161,73	30
274	Ani-LG-106	86919	2474129	99,42	64
275	Ani-LG-107	209759	2282759	183,40	46
278	Ani-LG-125	162272	2679921	53,28	48
283	Ani-LG-134	294609	2711520	155,27	24
286	Ani-LG-137	418021	2242477	100,03	23
290	Ani-LG-141	163664	2438536	90,50	35
293	Ani-LG-156	432341	2783493	96,03	21
295	Ani-LG-159	154078	2741724	105,22	34
296	Ani-LG-162	370362	2223869	85,70	20
299	Ani-LG-171	150380	2738387	67,81	33
300	Ani-LG-173	112538	2738267	83,17	34

301	Ani-LG-174	96084	2740955	90,82	49
	average	243011,70	2612861,31	150,84	38,90
	median	220096,50	2678970,00	157,51	35,00

ISBN: 978-82-575-1832-5
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

111345 / ANDVORDGRAFISK.NO