

TOWARDS A BETTER UNDERSTANDING OF THE EPIDEMIOLOGICAL SUCCESS OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

Valérie O. Baede

The background of the cover is a textured, light brown paper. It features large, overlapping wavy shapes in shades of red and orange. Interspersed among these are several sets of concentric, wavy lines in white and yellow. There are also clusters of circles in yellow and orange, some of which are solid and others are outlined. The overall aesthetic is modern and graphic.

**Towards a better understanding of
the epidemiological success of methicillin-resistant
*Staphylococcus aureus***

Valérie O. Baede

Colophon

ISBN: 978-94-6469-281-5

Cover design: Simone Golob

Layout design: Valérie Baede

Printing: Proefschriftmaken

Copyright © V.O. Baede

All rights reserved. No part of this publication may be reproduced, distributed, stored in a retrieval system, or transmitted in any form or by any means, without prior written permission from the author or, where appropriate, from the publishers of the corresponding journals.

The work presented in this thesis was financially supported by JPIAMR and ZonMw grant number 547001006.

Printing of this thesis was financially supported by:

SYMCEL 

**Towards a Better Understanding of the Epidemiological Success
of Methicillin-Resistant *Staphylococcus aureus***

Op weg naar een beter begrip van het epidemiologische succes
van meticilline-resistente *Staphylococcus aureus*

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 24 mei 2023 om 15:30 uur

door

Valérie Olga Baede

geboren te Geldrop

Promotiecommissie:

Promotor: prof.dr. M.C. Vos

Overige leden: prof.dr. S.G.M.A. Pasmans
prof.dr. J.A.J.W. Kluytmans
dr. E.M. Broens

Copromotor: dr. W.J.B. van Wamel

Contents

Chapter 1	
General introduction	7
Chapter 2	
Markers of epidemiological success of methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) isolates in European populations	31
Chapter 3	
MRSA surveillance programmes worldwide: moving towards a harmonised international approach	55
Chapter 4	
Dehydration tolerance in epidemic versus nonepidemic MRSA demonstrated by isothermal microcalorimetry	73
Chapter 5	
The survival of epidemic and sporadic MRSA on human skin mimics is determined by both host and bacterial factors	93
Chapter 6	
Nasal microbiome disruption and recovery after mupirocin treatment in <i>Staphylococcus aureus</i> carriers and noncarriers	111
Chapter 7	
Summarising discussion and future work	127
Chapter 8	
English summary	139
Chapter 9	
Nederlandse samenvatting	145
Appendices	
Dankwoord	153
Curriculum vitae	157
List of publications	159
PhD portfolio	161

Chapter 1

General Introduction

Methicillin-resistant *Staphylococcus aureus*, the cause of a great global health burden

Staphylococcus aureus (*S. aureus*) is a commensal bacterium residing on human skin and mucous membranes. As an opportunistic pathogen, it can cause a wide variety of superficial and invasive infections, potentially leading to sepsis ^{1,2}. *S. aureus* is the most frequently isolated pathogen in complicated skin and soft tissue infections ^{3,4}, vertebral osteomyelitis ⁵⁻⁸ and infective endocarditis ⁹. It is also the second most common cause of bloodstream infections (BSIs), with an annual incidence rate of 26,1 per 100.000 population ¹⁰, and the most important cause of BSI-associated death ¹¹⁻¹³.

Patients affected by *S. aureus* infections are treated with antimicrobial drugs. Usage of antimicrobial drugs is one of the causes for an increase in antimicrobial resistance. In *S. aureus*, the acquisition of resistance against penicillin and methicillin was demonstrated in the 1940s and the 1960s respectively, both shortly after the introduction of these drugs in the clinic ^{14,15}. *S. aureus* acquired methicillin resistance by the uptake of the *mecA* gene carried on a mobile genetic element: the staphylococcal cassette chromosome (SCC*mec*). The *mecA* gene encodes for the production of a modified penicillin-binding protein (PBP); PBP2a. Native PBPs play a role in cell wall synthesis through the cross-linking of peptidoglycan. They have a high affinity for beta-lactam antibiotics, which inactivate PBPs upon binding, thereby blocking cell wall synthesis. In contrast to native PBPs, PBP2a has a low affinity for beta-lactams, which means that most beta-lactam antibiotics, such as methicillin, are unable to bind PBP2a and thus unable to block cell wall synthesis ¹⁶. As a result, the bacteria are resistant to beta-lactam antibiotics.

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) complicates successful treatment. Already, this has led to a significant healthcare burden. In Europe, MRSA was the second most prevalent cause of infections and attributable deaths due to antimicrobial resistant pathogens in 2015 ¹⁷. In the USA, MRSA accounted for 10.600 estimated deaths and 1,8 billion USD attributable healthcare costs in 2017 ¹⁸. Globally, MRSA was the pathogen-drug combination with the highest number of attributable deaths (> 100.000) in 2019 due to bacterial antimicrobial resistance, meaning > 100.000 deaths could have been prevented if these infections were caused by methicillin-susceptible *S. aureus* (MSSA) ¹⁹. Furthermore, MRSA was responsible for 3,5 million disability-adjusted life years (DALYs) attributable to resistance in 2019 ¹⁹.

Over time, many MRSA strains have acquired resistance against older and newer classes of antibiotics as well, such as tetracyclines, aminoglycosides and also vancomycin ²⁰. Many of these resistance determinants are carried on mobile genetic elements in the staphylococcal genome, such as plasmids and transposons, enabling acquisition of resistance genes through horizontal gene transfer. As a result, antimicrobial resistance is a dynamic process, in which bacteria can rapidly acquire and subsequently lose resistance genes. Combined with an extensive arsenal of virulence factors, the ability of MRSA to evolve rapidly alongside clinical developments and to acquire resistance against multiple antimicrobial classes has driven its evolutionary success ²⁰. Already, MRSA is one of the most important pathogens in the global burden of bacterial antimicrobial resistance ¹⁹. Without proper interventions, we can expect this burden will only increase in the following decades.

How to reduce the burden of MRSA?

To mitigate the MRSA burden, much work is done to promote prudent use of antimicrobials and to develop new antimicrobial therapies. However, as mentioned above, the ability of MRSA to rapidly acquire resistance to new antimicrobial drugs sabotages some of these developments. Alternative therapies are also being developed, such as antimicrobial peptides and phage therapy. Nevertheless, the developments of these therapies are slow and it is still unknown whether these therapies will be successful and useful for treatment in patients. Therefore, to mitigate the MRSA burden successfully, the prevention of MRSA transmission and subsequent infections continues to be essential.

In hospitals, infection prevention and control measures are focused around hand hygiene, cleaning and disinfection, contact precautions and isolation, targeted screening at admission and decolonisation of carriers and/or patients prior to an at-risk intervention ^{20,21}. In the Netherlands, a strict infection prevention policy is implemented in hospitals; the search and destroy (S&D) policy. This policy comprises routine screening of all patients and healthcare workers (HCW) at risk for MRSA acquisition, pre-emptive isolation of patients screened at hospital admission, strict isolation of MRSA-positive patients and decolonisation of MRSA carriers ^{22,23}. These measures have been successful in the containment of MRSA infections and carriage and it is likely that they have contributed to the nationwide low MRSA prevalence in the Netherlands ^{23,24}.

Surveillance is another essential pillar of infection prevention. On the hospital or regional level, surveillance is important to monitor the effect of implemented infection control measures. However, surveillance is also crucial to gain a comprehensive understanding of MRSA epidemiology on the national or global level, by monitoring MRSA prevalence and identifying outbreaks and emerging MRSA clones.

An epidemiological overview of MRSA

MRSA is reported worldwide, but rates differ significantly between countries. On all continents, countries are found with a prevalence above 25% and this even exceeds 50% in some Asian and Latin American countries ^{20,25–27}. In Europe, MRSA prevalence historically ranges between 25–50% in Southern countries, while North-western countries generally report very low prevalence (<5%) ^{20,27–30}.

Looking at the epidemiological behaviour of MRSA, we can distinguish between 3 different types. The first type of MRSA to emerge was healthcare-associated MRSA (HA-MRSA), which caused outbreaks in hospitals around the world. Patients contracting HA-MRSA are often suffering from one or more comorbidities ³¹. In the 1990s, more and more MRSA cases appeared in the general community. This community-acquired MRSA (CA-MRSA) can occur in healthy individuals, but is also frequently associated with skin and soft tissue infections ^{31,32}. Lastly, contact with pigs, cattle or poultry is a major risk factor for acquiring livestock-associated MRSA (LA-MRSA) ^{33,34}.

A way to distinguish different MRSA strains is by the use of molecular typing techniques (Box 1). With these techniques, we can study the genetic evolution of MRSA and gain insight into the emergence of MRSA types as described above. Using multilocus sequence typing (MLST) and *SCCmec* typing, Enright *et al.*, showed that different *SCCmec* types were acquired by MRSA isolates of the same

Box 1**MRSA genotyping and nomenclature**

Historically, MRSA clones were often named after the place where they were discovered, e.g., the Brazilian clone. Over time, a lot of different genotyping techniques have been developed and used to study the molecular epidemiology of MRSA, including pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing *SCCmec* typing, multiple locus variable-number tandem repeat analysis (MLVA) and recently whole genome sequencing (WGS), among others. A description of these techniques is outside the scope of this thesis, but detailed information can be found in ^{41–47}. Due to the wide range of genotyping techniques developed for MRSA characterisation, a large variety in MRSA nomenclature exists. To understand the nomenclature of global MRSA clones, the following terms are most important. MLST allelic profiles are indexed in sequence types (STs). These STs cluster phylogenetically into clonal complexes (CCs). *SCCmec* types are distinguished by numbering in roman numerals (e.g., *SCCmec*-IV). PFGE was historically used to describe clones in the USA (e.g., USA300 or USA400). With the development of WGS, many other genotyping techniques have become redundant because their results can be derived from the WGS result ⁴⁸. WGS also enables simultaneous analysis of virulence and resistance genes present in the genome. While this provides many benefits, consensus is needed on bioinformatic analyses, data sharing and a new global and universal MRSA nomenclature to improve the epidemiological study of MRSA.

sequence type (ST), indicating *SCCmec* had been acquired on multiple occasions by strains of the same genetic background ^{35,36}. Additionally, it was shown that MRSA emerged as a limited number of clonal complexes (CCs), most importantly CC5, CC8, CC22, CC30 and CC45 ^{35,36}. These findings coincide with the earliest global molecular epidemiology studies, which showed that the majority of MRSA cases were attributable to only a handful of genetic lineages, named the Iberian (CC8-ST247-I), Brazilian (CC8-ST239-III), Hungarian (CC8-ST239-III), New York/Japan (CC5-ST5-II) and Paediatric (CC5-ST5-IV) MRSA clones ^{27,37}. Subsequent molecular epidemiological studies have shown how these clones have spread to a pandemic level and are still dominant today. Excellent overviews of pandemic and epidemic clones are given in ^{27,31,38–40}.

Crucial to the widespread dissemination of these clones is their ability to transmit successfully. However, not every MRSA clone is able to spread on a pandemic or even national level. And occasionally, a dominant MRSA clone in a certain geographic area is replaced by another MRSA clone, referred to here as clonal replacement. Some historic events of clonal replacement are described in Box 2.

These events demonstrate that MRSA transmission is not always successful. Some MRSA strains are only found sporadically. Aires de Sousa *et al.*, analysed minor clones and sporadic isolates found in single hospitals or only a few patients from single hospitals ⁴⁹. The authors concluded that most sporadic MRSA isolates evolved from epidemic clones. This finding indicates that while epidemic and sporadic strains can originate from the same genetic lineage, epidemic strains differ from sporadic strains in characteristics that promote successful transmission or happen to evolve in local niches that enhance successful transmission, either through environmental conditions that benefit transmission or higher susceptibility of the host.

To get a better understanding of the dynamic nature of MRSA epidemiology, more knowledge on the factors contributing to MRSA transmission success is needed to help prevent MRSA transmission and subsequently reduce the number of MRSA infections. In this thesis, we aim to identify intrinsic or external factors that lead to MRSA transmission success. First, known factors that potentially explain transmission success are described.

Box 2**Historic events of clonal replacement**

The MRSA epidemic in the UK has been historically dominated by two major clones, EMRSA-15 (CC22/ST22) and EMRSA-16 (CC30/ST36)⁵⁰. Monitoring of bacteraemia isolates showed a proportional decrease of EMRSA-16 with a simultaneous increase of EMRSA-15, indicating a shift in dominance from EMRSA-16 to EMRSA-15 between 2001 and 2007⁵¹. Today, EMRSA-15 is still the dominant MRSA clone in the UK. It subsequently spread throughout Europe and the world, including Germany, the Czech Republic, Portugal, New Zealand, Australia and Singapore⁵².

Before EMRSA-15 was introduced into Portugal, several shifts in MRSA dominance had already occurred^{53,54}. Halfway the 1980s, the Portuguese clone (PFGE type C, ST239-III) was the main clone in Portugal. In 1992-1993, it was replaced by the Iberian clone (PFGE type A, ST247-IA) and a second shift occurred a few years later, when the Brazilian clone (PFGE type B, ST239-III/IIIA) was introduced⁵³. The Brazilian clone spread rapidly throughout Portugal, becoming the major clone between 1998-2000. However, it got replaced by pandemic clone EMRSA-15 in the early 2000s, simultaneously with the clonal shifting in the UK. In 2005, another pandemic clone was first reported in a Portuguese hospital, namely the New York/Japan clone⁵⁵.

Other examples of clonal replacement in Europe are documented in the Czech Republic, Germany and Hungary. In Czech hospitals, the Brazilian clone (ST239-III) accounted for 80% of MRSA isolates in 1996-1997, next to 12% isolates of the Iberian clone (ST247-IA)⁵⁶. During 2000-2002, the Brazilian clone was replaced by another ST239-III clone, named the Czech clone, next to persistence of the Iberian clone⁵⁷. EMRSA-15 emerged to Czech Republic around 2003⁵⁸. In the late 1990s in Germany, 4 different clones of 3 clonal complexes were dominating hospitals⁵⁹. Besides the Northern German clone ST247-I (CC8) and the Hannover epidemic clone ST254 (CC8), the South German clone ST228-I (CC5) and the Berlin clone ST45-IV (CC45) were frequently found. Isolation of EMRSA-15 was already reported in 1998 and this clone became dominant in 2002 (here also called the Barnim epidemic MRSA). Alongside EMRSA-15, the New York/Japan (Rhine-Hesse) clone ST5-II (CC5) emerged. Also in Hungary, a series of replacements were demonstrated⁶⁰. The ST239-III Hungarian clone was the predominant clone in the 1990s. In 1997-1998, it was accompanied by the ST-228-I South German clone, which became the dominant clone in the early 2000s next to the ST5-II New York/Japan clone which emerged in 2001⁶⁰.

In various Asian countries, ST239-III was dominant prior to 2000^{61,62}. This was also the case in Singapore, until ST22 (EMRSA-15) was isolated for the first time in 2003 and became the dominant lineage in Singaporean hospitals⁶². In Taiwan, significant increases of ST59 and ST5 led to a proportional decrease of ST239 between 2000-2010⁶³.

Between 1995-2003, USA100 (New York/Japan clone) and USA200 (EMRSA-16) were the most common MRSA clones isolates from healthcare-associated infections in the United States⁶⁴. In the community, USA400 (CC1) was replaced by USA300 (CC8) after 2001³².

Known factors potentially explaining MRSA transmission success

Many factors can benefit MRSA transmission and potentially play a role in a clone's success or replacement. We discuss these factors considering their association with the pathogen, the environment and the host. Additionally, we look at external factors influencing the host-pathogen interaction, focusing on infection prevention policy and management.

Pathogen*Genetic characteristics of S. aureus contributing to transmission success*

S. aureus pathogenicity is attributed to its large variety of toxins, other virulence factors and antimicrobial resistance (AMR) genes. These characteristics of *S. aureus* are encoded in its genome. The staphylococcal genome is made up of a core genome, a core-variable region and mobile genetic elements (MGEs). The

core genome carries many essential genes involved in metabolism, housekeeping and replication among other fundamental functions⁶⁵. The core-variable region consists of a unique combination of genes that are highly conserved within *S. aureus* lineages but unique compared to other lineages⁶⁶. Many of these genes encode for virulence factors such as surface-attached proteins and immune evasion proteins that are essential for host-pathogen interaction^{66,67}. Nevertheless, isolates from the same lineage can demonstrate very different epidemiological behaviour. This suggests that the success of strains with more invasive and/or epidemic behaviour is not supported by the core-variable region of the genome, but might be supported by the highly variable MGEs⁶⁸. MGEs carry genes involved in host-adaptation, virulence and AMR. The transfer of MGEs through horizontal gene transfer enables individual *S. aureus* isolates to adapt quickly to new niches⁶⁸, which potentially supports successful transmission.

Virulence factors

An example of MGEs carrying genes involved in the host-pathogen interaction of *S. aureus* are bacteriophages. Bacteriophages of the $\phi 3$ family carry a so-called immune evasion cluster, encoding for multiple immune evasion proteins including chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN) and staphylokinase (SAK)⁶⁹. These $\phi 3$ phages are found in multiple *S. aureus* lineages, indicating they play an important role during survival in the human host⁷⁰. Indeed, they are carried by HA-MRSA as well as CA-MRSA clones and recently also emerged in LA-MRSA isolates from human infections without livestock contact^{68,71}. Nevertheless, carriage of the immune evasion cluster is associated with both colonisation and infection isolates, so its role in transmission success is still unclear⁶⁶. Bacteriophages of the $\phi 2$ family are also important in *S. aureus* virulence, as they can carry *lukFS-PV* which encodes for Pantone-Valentine leucocidin (PVL), a pore-forming toxin⁷². This toxin is strongly associated with skin and soft tissue infections caused by CA-MRSA clones⁷³. However, PVL is not produced by all CA-MRSA clones and also not exclusively related to the success of some CA-MRSA clones⁷³. Various studies have argued that the epidemic success of USA300 is related to the uptake of the arginine catabolic mobile element (ACME), encoding for virulence genes *arc*, *sasX* and *speG*^{74–78}. The ACME *arc* gene contributes to survival in acidic environments such as in sweat⁷⁶. The ACME *sasX* gene enhances nasal colonisation, abscess formation and tissue damage⁷⁷. The ACME *speG* gene enables tolerance to lethal polyamines that are produced by human tissues in response to inflammation⁷⁴. In USA300, *speG*-mediated tolerance allows for increased biofilm formation, increased fibrinogen and fibronectin binding and decreased killing by human keratinocytes⁷⁴. In a rabbit bacteraemia model, deletion of ACME led to reduced fitness⁷⁵. However, presence of ACME was not associated with USA300 virulence in a rat pneumonia model or murine skin model⁷⁹. A more recent study found no differences in survival on murine skin and also bacterial invasion, intracellular replication and cytotoxicity in a human lung epithelial model was similar for the wild-type USA300 strain and an ACME mutant⁸⁰. Other virulence factors encoded on MGEs could support MRSA transmission as well. Exfoliative toxin A and B are encoded on a bacteriophage and plasmid respectively^{81,82}. They cause breakages in keratinocyte junctions leading to blistering of the skin and are associated with staphylococcal scalded skin syndrome (SSSS)⁷². A recent study showed that ϕ ETA is relatively stable within lineages and suggests that the recent increased incidence of SSSS in North America is associated with migration and expansion of existing lineages⁸³. Nevertheless, it is unknown if ϕ ETA is an actual driver of transmission.

Many virulence factors, such as the ones mentioned here, have been suggested as drivers of transmission success. However, while the presence of certain virulence factors might support transmission success of some clones, they do not always explain the successful spread of others. Potential explanations are that (i) a synergistic combination of virulence factors leads to transmission success, (ii) different (combinations of) virulence factors lead to transmission success in different clones or (iii) the main virulence factor supporting transmission success is not identified yet. This last explanation seems unlikely, as one main success factor for all epidemic clones should be clearly distinct. **To understand the role of virulence factors in relation to transmission of successful versus unsuccessful MRSA clones, their presence in successful and unsuccessful clones should be evaluated in relation to other virulence factors and other unrelated clones.**

Resistance determinants

Genes encoding resistance to antimicrobial drugs are often carried on MGEs as well. The most relevant AMR gene for MRSA is the *mecA* gene, which is carried on a SCC element and leads to methicillin resistance as described above. Multiple types of SCCmec elements are classified. Several SCCmec (sub)types carry integrated copies of other MGEs harbouring resistance genes, such as plasmid pUB110 which encodes for resistance against aminoglycosides (*ant4*) and glycopeptide bleomycin (*ble*)^{84,85}. Another plasmid, pT181, encoding for tetracycline resistance (*tetK*) is integrated into SCCmec type III^{85,86}. Examples of transposons carried on SCC are Tn554, encoding for spectinomycin (*ermA*, aminoglycoside) and erythromycin (*aad9* or *spc*, macrolide) resistance, and Tn4001, encoding for aminoglycoside resistance (*aacA-aphD*)^{84,85}. The *mec* gene complex in SCCmec type IX also contains beta-lactamase encoding gene *blaZ*⁸⁵.

AMR genes contribute to the transmission success of successful MRSA clones. HA-MRSA clones are usually multidrug resistant⁸⁷. Important for HA-MRSA is the resistance to fluoroquinolones, such as ciprofloxacin, demonstrated by mutations in *gyrA* and *parC* genes necessary for successful spread of HA-MRSA clone CC22 in the UK⁵². Ciprofloxacin resistance in HA-MRSA was likely stimulated by the epidemic expansion of major HA-MRSA clones CC22 and CC30 after which CC22 outcompeted the former due to its increased growth rate and fitness⁸⁸. Resistance against aminoglycosides and macrolides is important in HA-MRSA as well⁶⁸. For ST239, increased resistance to glycopeptides and daptomycin was acquired, but was accompanied by reduced fitness and attenuated virulence⁸⁷. CA-MRSA were initially susceptible to most antimicrobials, but various successful clones have acquired resistance against mupirocin, macrolides, lincosamides, fluoroquinolones, tetracyclines or fusidic acid⁸⁹. LA-MRSA is characterised by resistance to tetracyclines (*tet(M)* and *tet(K)*), which is potentially driven by the use of these drugs in livestock³³. Furthermore, LA-MRSA isolates can carry resistance determinants against macrolides (*erm(A)*, *erm(B)* and *erm(C)*), lincosamides (*Inu(A)*) and aminoglycosides (*aacA-aphD*, *aadD*), next to a few resistance genes uncommon in staphylococci, such as *fexA* (fenicols), *dfrrK* (trimethoprim), *vga(C)* and *vga(E)* (pleuromutilins-lincosamides-streptogramins)⁹⁰.

From these observations, it is clear that different types of MRSA are associated with certain resistance genes. These genes likely supported transmission success. However, as for virulence genes, more information is needed to decipher which genes are the driving factors of transmission success and which are accidental

hitchhikers carried on the same MGE. Again, as for virulence factors, it is possible that different (combinations of) resistance genes drive transmission of different successful clones. **To get a complete understanding of the relative effect of resistance genes on the transmission success of MRSA, the association of these genes with epidemic behaviour of MRSA should be investigated in relation to virulence determinants.**

Environment

Bacterial transmission in relation to bacterial survival on surfaces and fomites.

Before colonisation or infection can occur, the host needs to come into contact with *S. aureus*. Acquisition of this bacterium can occur through direct and indirect transmission routes. A direct transmission route might be skin-to-skin contact. Indirectly, *S. aureus* can be dispersed into the environment, through sneezing or shedding of colonised skin particles ^{2,91}. The most common indirect transmission route is through contamination of hands after picking or touching the nose ². In 90% of carriers, *S. aureus* is found on the hands ². Contamination of hands will often lead to contamination of surfaces and objects (fomites), which will form a reservoir for *S. aureus* acquisition in hospitals and households. In hospitals, MRSA has been found on floors, bed linens, patient gowns, blood pressure cuffs, computer keyboards, faucet handles and more ^{92–94}. In households, MRSA has been found on bed linens, towels, light switches, door knobs, television remote controls, computer keyboards and more ^{95,96}.

S. aureus, also MRSA, is tolerant to a wide range of temperatures, humidity and dehydration, which supports its ability to survive on surfaces and fomites for > 6 months during which they can be acquired by a new host ^{91,97}. Various studies have suggested that epidemic MRSA are more tolerant to dehydration, leading to an increased capability to survive in the environment and thus increased risk of transmission ^{88,98–101}. Knight *et al.* found that EMRSA-15 (CC22) had an increased tolerance to dehydration in comparison to EMRSA-16 (CC30) and ST239 ⁸⁸. Unfortunately, most of these studies looked at a limited number of isolates or clones and their results cannot be translated to the larger MRSA population. To study the influence of dehydration tolerance on transmission success of MRSA, a much larger and varied collection of epidemic and sporadic MRSA isolates should be investigated. Nevertheless, sensitivity of current methods to quantify bacterial survival is limited and insufficient to compare large numbers of strains. Therefore, **new robust tools are needed to study dehydration tolerance in relation to epidemiological success in large collections of MRSA strains.**

Host

The role of skin colonisation in transmission success

S. aureus carriage is common among humans. Approximately 20% (range 12–30%) of the general population are persistent carriers of *S. aureus*, while another 30% (16–70%) are intermittent carriers ². The most common carriage site of *S. aureus* in humans is the nose ². The nose forms a reservoir, from which other body sites can be colonised ². Examples of other frequently colonised body sites are the throat, skin and perineum ². *S. aureus* carriage is a risk factor for subsequent infection ¹⁰². This has been shown for a range of infections including bacteraemia ^{103,104} and surgical site infections ^{105–107}. Persistent carriers have a higher risk of developing infections than intermittent and noncarriers ^{2,103,104,108}.

For MSSA as well as MRSA, carriage on skin or in the nose is a risk factor for transmission to others or the environment ^{2,109}. Therefore, to explain the transmission success of epidemic MRSA clones, it is important to understand survival and carriage dynamics of *S. aureus* in the human nose and on human skin. It is still unclear what causes the difference between persistent and intermittent carriers and noncarriers and whether this is dependent on host or bacterial factors.

It is possible that persistent carriers are more susceptible to *S. aureus*, leading to easier and more frequent colonisation and subsequent transmission. To test the susceptibility of carriers to different *S. aureus* strains, several artificial inoculation studies have been done. When inoculated intranasally with a mixture of multiple *S. aureus* strains, including the resident strain for carriers, persistent carriers favoured colonisation with their own resident strain ^{110,111}. Persistent carriers were also more likely to get recolonised with their resident strain (58%) than intermittent carriers (17%) ¹¹¹. The majority of infections in persistent carriers are also caused by the patient's own *S. aureus* strain ^{103,104}. These studies indicate that carriage is not random, but the result of an optimal fit between host and colonising strain ^{110,111}. Therefore, it is likely that carriage is at least partially facilitated by host factors, which allow *S. aureus* to survive on epithelial cells of the human skin and mucous membranes. For this, *S. aureus* has to compete with other bacteria in the local microbiota and to fight local immune responses. It is therefore likely that these interactions play a role in *S. aureus* carriage and transmission.

Role of the host microbiome in S. aureus carriage

The nasal microbiota resembles that of the skin ¹¹². Seven distinct nasal microbiota composition profiles, so-called community state types (CSTs), could be defined in humans based on the proportional abundance of specific nasal bacteria. Each is dominated by a specific bacterial genus or species: *S. aureus* (CST1), Enterobacteriaceae (including *Escherichia* spp, *Proteus* spp and *Klebsiella* spp) (CST2), *S. epidermidis* (CST3), *Propionibacterium* spp (CST4), *Corynebacterium* spp (CST5), *Moraxella* spp (CST6) and *Dolosigranulum* spp (CST7) ¹¹³. Differences in the composition of the nasal microbiota have been found for *S. aureus* carriers compared to noncarriers ^{113,114}. In *S. aureus* carriers, relatively higher abundances of *Cutibacterium acnes*, *Corynebacterium accolens* and *S. epidermidis* were found compared to noncarriers, while *Corynebacterium pseudodiphtheriticum*, *Dolosigranulum* spp and *Cutibacterium granulosum* were less abundant in *S. aureus* carriers ^{113,114}. It is likely that the differences in microbiota composition are the result of competition for epithelial binding sites and nutrients ¹¹⁵. For example, *S. epidermidis* can secrete the extracellular serine protease (Esp), which degrades surface adhesins thereby preventing *S. aureus* colonisation ^{116,117}. On the other hand, *S. aureus* can produce approximately 10 different proteases for the breakdown of proteins in the nasal fluid, such as albumin and mucins, while other nasal bacteria produce none or only a few extracellular proteases, leading to an advantage in competition for nutrients ¹¹⁵. The production of antimicrobial molecules by *S. aureus* or other members of the nasal microbiota leads to active inhibition of microbial competitors ¹¹⁵. Carriage of *S. lugdunensis* was associated with a 6-fold lower risk of *S. aureus* carriage, possibly due to its production of lugdunin, an antimicrobial peptide, that kills *S. aureus* ¹¹⁸. *Streptococcus pneumoniae* produces H₂O₂, which leads to DNA-damaging radicals and activation of *S. aureus* prophages, leading to *S. aureus* cell lysis ^{119,120}. Besides naturally driven competition, *S. aureus* can also be removed from the nose artificially. To prevent autologous *S. aureus* infections, it is recommended to subject *S. aureus* carriers to a

decolonisation treatment before at-risk interventions ^{121,122}. This decolonisation procedure consists of topical treatment with mupirocin nasal ointment, often combined with a chlorhexidine cutaneous body wash. Mupirocin is a broad-spectrum antibiotic and will have an effect on multiple members of the nasal microbiota ¹²³, therefore alter the nasal microbiota and potentially influence *S. aureus* (re)colonisation through mechanisms described above. Together, **these findings show that the interaction between members of the nasal microbiota are complex and can play a crucial role in *S. aureus* carriage, making it important to understand the longitudinal effect of mupirocin treatment.**

*The role of host immunity in *S. aureus* carriage*

Both the epidermal layer of the skin, as well as the vestibulum nasi, the so-called 'nose-picking site', consists of keratinised stratified squamous epithelium ^{124,125}. This epithelium forms the first line of host defence against invading micro-organisms. The keratinocytes in this epithelium can express several pattern recognition receptors. Upon recognition of conserved microbial structures, such as peptidoglycan, these receptors trigger an innate immune response ¹²⁶. Toll-like receptor 2 (TLR2) is the main receptor for recognition of staphylococcal ligands, such as lipoproteins ^{127,128}. Upon activation of pattern recognition receptors or disruption of the epithelial structure, keratinocytes produce pro-inflammatory cytokines, such as interleukin (IL)-1 β , and antimicrobial peptides (AMPs), such as human beta-defensin (hBD)-2, hBD-3 and RNase 7 ^{126,129,130}. In turn, these AMPs trigger the production of IL-6 and chemokine CXCL8, as well as the attraction and activation of immune cells, such as dendritic cells, neutrophils and T cells ^{130,131}. Additionally, AMPs have bacteriostatic or bactericidal activity against *S. aureus* ¹³²⁻¹³⁴.

There are some indications that *S. aureus* carriers have a different innate immune response than noncarriers, such as illustrated by the significantly higher baseline levels for IL-1 β and IL-6 in carriers ¹³⁵. Gene polymorphisms in the β -defensin promotor region (DEFB1) were associated with reduced expression of hBD-1 and hBD-3 in skin, which was associated with persistent carriers ¹³⁶. Despite these and other immune gene polymorphisms that are associated with higher risk for *S. aureus* nasal carriage, genetic correlations do not ultimately explain persistent *S. aureus* carriage ¹³⁷. Nevertheless, variation in host immunity might facilitate different levels of survival of MRSA strains on human skin. In case of successful survival, this can be followed by sustained carriage or infection and subsequent transmission to others. Variation in host immunity might therefore promote MRSA transmission success in certain human populations. **To elucidate the role of host immunity in transmission success, the interaction between the local immunity of the host and epidemic or sporadic isolates of MRSA during skin infection should be studied.**

Policy

Besides intrinsic factors, external factors like health policy and management can influence transmission of infectious diseases or our view on this transmission. Here, we focus on surveillance, infection prevention and control and usage of antimicrobial drugs.

Surveillance

As described above, surveillance is an important tool to monitor MRSA prevalence and evaluate the effect of implemented control measures. For MRSA, surveillance

programmes have been established on the institutional, regional, national and even multinational level. One example of a multinational surveillance programme is the European Antimicrobial Resistance Surveillance Network (EARS-Net), accommodated by the European Centre for Disease Prevention and Control (ECDC). Through this network, surveillance data are gathered for bloodstream infections (BSI) caused by MRSA in participating EU/EEA countries. These data are used to compare MRSA prevalence in European countries. Figure 1 shows MRSA prevalence as percentage of invasive *S. aureus* isolates with resistance to methicillin in EU/EEA countries in 2009, 2014 and 2019. The differences in MRSA prevalence between Northern/North-western and Southern countries are quite clear (Figure 1). Between 2009 and 2019, multiple countries were able to reduce MRSA prevalence (Figure 1).

Interpretation of the available prevalence data is difficult. Most prevalence data result from individual research studies, which differ largely in methodology and target populations (healthcare-associated infections versus community-acquired infections, invasive versus non-invasive infections etc). Systematic data collection on a multinational level is scarce. And despite its systematic approach, even the data collection through EARS-Net has its limitations for monitoring MRSA prevalence. First, MRSA prevalence is calculated as the percentage of *S. aureus* isolates with methicillin resistance. However, when *S. aureus* prevalence differs between countries as well, it is difficult to compare the actual impact of MRSA. Second, data collection is restricted to invasive isolates, while emerging CA-MRSA is less likely to cause invasive infections, therefore leading to a potential underestimation of MRSA prevalence. Third, multinational programmes are dependent on regional or national programmes for data input. Differences in data collection between these regional and national programmes lead to potential misinterpretation of the data. **To tackle these challenges in MRSA surveillance, it is important to strive for harmonisation in MRSA surveillance programmes.** This will also lead to higher quality data for research, which will help us to identify factors that influence MRSA transmission.

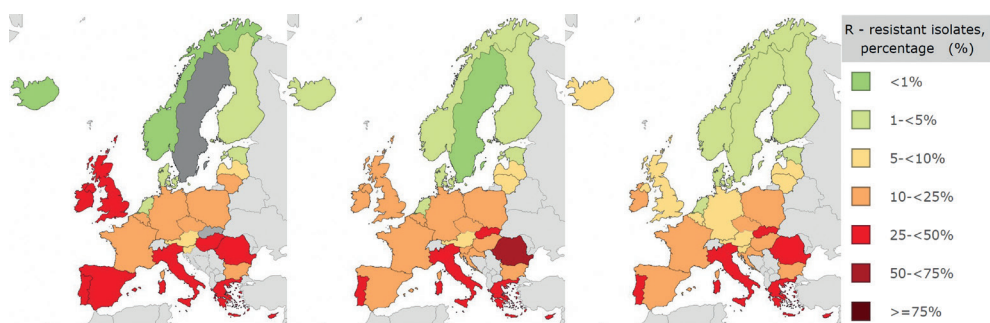


Figure 1. MRSA prevalence in European countries in 2009, 2014 and 2019.

MRSA prevalence given as percentage of invasive *S. aureus* isolates with resistance to methicillin in EU/EEA countries. ECDC Surveillance Atlas of Infectious Diseases. Dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries.*

* The views and opinions of the authors expressed herein do not necessarily state or reflect those of the ECDC. The accuracy of the authors' statistical analysis and the findings they report are not the responsibility of ECDC. ECDC is not responsible for conclusions or opinions drawn from the data provided. ECDC is not responsible for the correctness of the data and for data management, data merging and data collation after provision of the data. ECDC shall not be held liable for improper or incorrect use of the data.

Infection prevention policies

As described above, MRSA prevalence can differ widely in countries despite similar geographical, societal and economic circumstances. A potential explanation for these differences in MRSA prevalence are the historical and regional differences in MRSA control management. MRSA was first detected in 1960¹⁵. The first national guidelines in Europe on MRSA prevention and control were issued in the 1970s and 1980s, by Sweden and the Netherlands respectively¹³⁸. Similar to the Netherlands, the Nordic countries implemented a strict infection prevention policy. Empirical evidence from observational studies and mathematical models suggests the effectiveness of this policy, resulting in the continuous low MRSA prevalence in these countries²³. In the United Kingdom, MRSA hospital outbreaks in the 1980s led to the implementation of a S&D approach in affected hospitals¹³⁹. However, overwhelming numbers of infections due to the epidemic behaviour of the involved MRSA strains (EMRSA-15 and EMRSA-16) made adherence to the S&D guidelines unfeasible¹³⁹. A steady increase in infections followed and the total number of MRSA BSIs peaked in 2003-2004¹³⁹. In 2004, the UK government announced a mandatory target to reduce the number of MRSA BSIs by 50% in four years¹³⁹. A long list of guidelines was published, contributing to an increased focus on infection prevention and this led to a successful decrease in MRSA BSI. By 2009-2010 this reduction was already 75% and has still declined over the last decade (Figure 1)¹³⁹. In France, implementation of general guidelines for infection control was coordinated on a regional level from 1992 onwards. Before the introduction of a new infection control program on a national level in 2005-2008, these regional guidelines had already led to an increase in the number of hospitals with defined action plans for prevention and surveillance of nosocomial infections, besides an overall decrease of MRSA BSI¹⁴⁰. In other Southern European countries, such as Italy, there is still significant opportunity to improve MRSA control¹⁴¹. In 2009, only one-third of Italian hospitals had written MRSA control guidelines and only one-eighth of hospitals had well-organised MRSA control programmes implemented¹⁴¹. The lack of appropriate guidelines in Italy is reflected by the high MRSA prevalence as reported by EARS-Net (Figure 1). These observations illustrate that appropriate infection prevention and control measures are important to mitigate MRSA transmission and can effectively reduce the number of MRSA infections. Therefore, **the local policies on infection prevention and control are an important factor in MRSA transmission success.**

Antimicrobial drug usage and selective pressure

The use of antimicrobial drugs supports the selection of the fittest bacteria i.e., the ones that are resistant against these drugs, whether this is intrinsic or acquired resistance. For patients, exposure to antibiotics is a risk factor for the acquisition of MRSA²⁰. Also on a larger level, antimicrobial usage drives AMR through selective pressure^{20,142}. For several MRSA clones, resistance to certain drugs may have contributed to their success, such as fluoroquinolone resistance for EMRSA-15 (CC22) clone^{52,88} and tetracycline resistance in LA-MRSA CC398¹⁴³.

The actual emergence of MRSA was likely driven by the use of penicillin. WGS studies have shown that the *mecA* gene was acquired in the 1940s, simultaneously with the introduction of penicillin in clinical practice¹⁴⁴. The acquisition of *mecA* next to *blaZ* provided additional resistance to beta-lactams which likely supported the emergence of MRSA, even before methicillin was introduced in practice¹⁴⁴. This demonstrates that usage of antimicrobial drugs does not only drive direct

resistance to that single compound, but can also result into indirect resistance to other drugs of the same class (cross-resistance) or other classes (co-resistance). Therefore, **usage of antimicrobial drugs and the resulting selective pressure can be important factors in transmission and should be accounted for in the study of transmission success.**

Main aim and outline of this thesis

In order to prevent MRSA infections, it is crucial to understand the drivers of MRSA transmission. **The main aim of this thesis is to investigate bacterial, host and environmental factors that might explain transmission success of epidemic MRSA strains, in contrast to sporadic MRSA strains.** The work is focused on MRSA originating from 3 European countries with distinct MRSA epidemiology despite similar geographical, societal and economic characteristics: France, the Netherlands and United Kingdom.

The Netherlands presents the overall lowest MRSA prevalence of the three countries (Figure 2). It has an extensive mandatory surveillance programme for MRSA, which has demonstrated a relatively large diversity of MRSA clones within the country. Between 2010 and 2020, MLVA complexes (MC) MC0398, MC0005, MC0008, MC0022, MC0045 and MC0030 were most common, together accounting for > 80% of MRSA isolates^{145,146}. These MLVA-MCs correspond to MLST-CCs CC398, CC5, CC8, CC22, CC45 and CC30 respectively. A different situation is found in the United Kingdom. Here, levels of MRSA prevalence used to be high due to epidemic waves of two major MRSA clones⁵⁰. From 2000 on, a large reduction in MRSA prevalence was seen from > 45% to < 10% (Figure 2). The majority of MRSA isolates belong to CC22 (77%), while CC30 (14%), CC1 (1%), CC5 (3%) and CC8 (2%) are found less frequent¹⁴⁷. In France, MRSA prevalence has decreased from 33% to 12% between 2001 and 2020 (Figure 2). The molecular epidemiology of MRSA in France is dominated by the Lyon clone (ST8-IV) (69%)¹⁴⁸. Other minor clones are the Paediatric clone (ST5-IV), its variant (ST5-VI), the Geraldine clone (ST5-I) and the European clone (ST80-IV), each accounting for 3-8% of MRSA isolates¹⁴⁸.

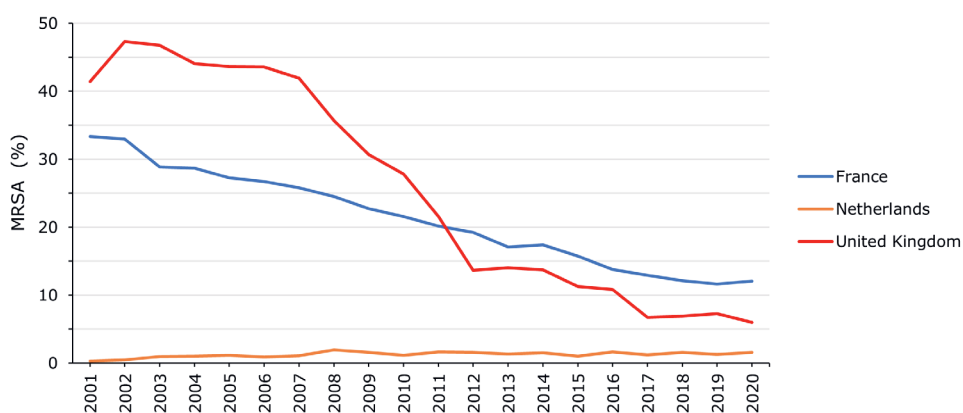


Figure 2. MRSA prevalence in France, the Netherlands and the United Kingdom, period 2001-2020.

MRSA prevalence given as percentage of invasive *S. aureus* isolates with resistance to methicillin in EU/EEA countries. ECDC Surveillance Atlas of Infectious Diseases.

Combined, epidemiological data of these three countries present information on multiple epidemic clones showing different behaviour in countries with historically different infection prevention policies, providing the opportunity to compare potential success factors of these epidemic clones within and between countries.

The first part of this thesis describes the challenges of identifying epidemic and sporadic strains. In **Chapter 2**, we aim to develop a unified method for identification of epidemic and sporadic MRSA isolates in multiple countries and to compile a balanced collection of MRSA strains from France, the Netherlands and the United Kingdom. This strain collection is studied to identify antimicrobial resistance markers of transmission success, in relation to antibiotic usage in each country and bacterial population genetics. In **Chapter 3**, we focus on the underlying reasons for the difficulties in identification of epidemic and sporadic strains. For this, we study the diversity of MRSA surveillance programmes worldwide and propose a framework for standardised MRSA surveillance.

The second part of this thesis describes the ability of MRSA to survive in the environment under stressful conditions. In **Chapter 4**, the association between transmission success of MRSA and dehydration tolerance is investigated with a newly developed tool, using isothermal microcalorimetry in combination with bacterial growth modelling.

The last part of this thesis focuses on the interaction with the human host. In **Chapter 5**, the association between transmission success of MRSA and its ability to survive on human skin is investigated. Additionally, the production of bacterial virulence factors, immune evasion proteins and host immune factors during interaction with keratinocytes is evaluated. In **Chapter 6**, we study the longitudinal influence of *S. aureus*-targeted decolonisation treatment on the nasal microbiota in *S. aureus* carriers and noncarriers and we monitor subsequent recolonisation with *S. aureus* in these individuals.

References

1. Lowy, F. D. *Staphylococcus aureus* Infections. *N. Engl. J. Med.* **339**, 520–532 (1998).
2. Wertheim, H. F. *et al.* The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* **5**, 751–762 (2005).
3. Moet, G. J., Jones, R. N., Biedenbach, D. J., Stilwell, M. G. & Fritsche, T. R. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: Report from the SENTRY Antimicrobial Surveillance Program (1998–2004). *Diagn. Microbiol. Infect. Dis.* **57**, 7–13 (2007).
4. Dryden, M. S. Complicated skin and soft tissue infection. *J. Antimicrob. Chemother.* **65**, iii35–iii44 (2010).
5. Bhavan, K. P. *et al.* The epidemiology of hematogenous vertebral osteomyelitis: a cohort study in a tertiary care hospital. *BMC Infect. Dis.* **10**, 158 (2010).
6. McHenry, M. C., Easley, K. A. & Locker, G. A. Vertebral Osteomyelitis: Long-Term Outcome for 253 Patients from 7 Cleveland-Area Hospitals. *Clin. Infect. Dis.* **34**, 1342–1350 (2002).
7. Corrah, T. W., Enoch, D. A., Aliyu, S. H. & Lever, A. M. Bacteraemia and subsequent vertebral osteomyelitis: a retrospective review of 125 patients. *QJM* **104**, 201–207 (2011).
8. Beronius, M., Bergman, B. & Andersson, R. Vertebral Osteomyelitis in Göteborg, Sweden: A Retrospective Study of Patients During 1990–95. *Scand. J. Infect. Dis.* **33**, 527–532 (2001).
9. Fowler, V. G. *et al.* *Staphylococcus aureus* Endocarditis. *JAMA* **293**, 3012–3021 (2005).
10. Laupland, K. B. *et al.* The changing epidemiology of *Staphylococcus aureus* bloodstream infection: A multinational population-based surveillance study. *Clin. Microbiol. Infect.* **19**, 465–471 (2013).
11. Uslan, D. Z. *et al.* Age- and Sex-Associated Trends in Bloodstream Infection. *Arch. Intern. Med.* **167**, 834–839 (2007).
12. Madsen, K. M., Schønheyder, H. C., Kristensen, B. & Sørensen, H. T. Secular trends in incidence and mortality of bacteraemia in a Danish county 1981–1994. *APMIS* **107**, 346–352 (1999).
13. Laupland, K. B. *et al.* Severe bloodstream infections: A population-based assessment. *Crit. Care Med.* **32**, 992–997 (2004).
14. Kirby, W. M. M. Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. *Science*. **99**, 452–453 (1944).
15. Jevons, M. P. 'Celbenin' - resistant Staphylococci. *BMJ* **1**, 124–125 (1961).
16. Hartman, B. J. & Tomasz, A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J. Bacteriol.* **158**, 513–6 (1984).
17. Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* **19**, 56–66 (2019).
18. Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2019. U.S. Department of Health and Human Services, CDC. (2019).
19. Murray, C. J. *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 (2022).
20. Lee, A. S. *et al.* Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Prim.* **4**, 18033 (2018).

21. Calfee, D. P. *et al.* Strategies to Prevent Methicillin-Resistant *Staphylococcus aureus* Transmission and Infection in Acute Care Hospitals: 2014 Update. *Infect. Control Hosp. Epidemiol.* **35**, 772–796 (2014).
22. Werkgroep Infectiepreventie (WIP). Meticilline-resistente *Staphylococcus aureus* (MRSA). <https://www.rivm.nl/documenten/wip-richtlijn-mrsa-ziekenhuizen> (2012).
23. Larsen, J. *et al.* Preventing the introduction of methicillin-resistant *Staphylococcus aureus* into hospitals. *J. Glob. Antimicrob. Resist.* **2**, 260–268 (2014).
24. Vos, M. C. *et al.* 5 Years of Experience Implementing a Methicillin-Resistant *Staphylococcus aureus* Search and Destroy Policy at the Largest University Medical Center in the Netherlands. *Infect. Control Hosp. Epidemiol.* **30**, 977–984 (2009).
25. Song, J.-H. *et al.* Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *J. Antimicrob. Chemother.* **66**, 1061–1069 (2011).
26. Mejía, C., Zurita, J. & Guzmán-Blanco, M. Epidemiology and surveillance of methicillin-resistant *Staphylococcus aureus* in Latin America. *Brazilian J. Infect. Dis.* **14**, 79–86 (2010).
27. Stefani, S. *et al.* Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int. J. Antimicrob. Agents* **39**, 273–282 (2012).
28. European Centre for Disease Prevention and Control. *Antimicrobial resistance surveillance in Europe 2009. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)* (2010).
29. European Centre for Disease Prevention and Control. *Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)* (2015).
30. European Centre for Disease Prevention and Control. *Surveillance of antimicrobial resistance in Europe - Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2018.* (2019).
31. David, M. Z. & Daum, R. S. Community-Associated Methicillin-Resistant *Staphylococcus aureus*: Epidemiology and Clinical Consequences of an Emerging Epidemic. *Clin. Microbiol. Rev.* **23**, 616–687 (2010).
32. DeLeo, F. R., Otto, M., Kreiswirth, B. N. & Chambers, H. F. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* **375**, 1557–1568 (2010).
33. Fluit, A. C. Livestock-associated *Staphylococcus aureus*. *Clin. Microbiol. Infect.* **18**, 735–744 (2012).
34. van den Broek, I. V. F. *et al.* Methicillin-resistant *Staphylococcus aureus* in people living and working in pig farms. *Epidemiol. Infect.* **137**, 700–708 (2009).
35. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
36. Robinson, D. A. & Enright, M. C. Evolutionary Models of the Emergence of Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**, 3926–3934 (2003).
37. Oliveira, D. C., Tomasz, A. & de Lencastre, H. The Evolution of Pandemic Clones of Methicillin-Resistant *Staphylococcus aureus* : Identification of Two Ancestral Genetic Backgrounds and the Associated mec Elements. *Microb. Drug Resist.* **7**, 349–361 (2001).
38. Monecke, S. *et al.* A Field Guide to Pandemic, Epidemic and Sporadic Clones of Methicillin-Resistant *Staphylococcus aureus*. *PLoS One* **6**, e17936 (2011).
39. Bal, A. M. *et al.* Genomic insights into the emergence and spread of international clones of healthcare-, community- and livestock-associated methicillin-resistant *Staphylococcus aureus*: Blurring of the traditional definitions. *J. Glob. Antimicrob. Resist.* **6**, 95–101 (2016).

40. Lakhundi, S. & Zhang, K. Methicillin-Resistant *Staphylococcus aureus*: Molecular Characterization, Evolution, and Epidemiology. *Clin. Microbiol. Rev.* **31**, e00020-18 (2018).
41. Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J. & Spratt, B. G. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* **38**, 1008–15 (2000).
42. Stephens, A. J., Huygens, F. & Giffard, P. M. Systematic Derivation of Marker Sets for Staphylococcal Cassette Chromosome *mec* Typing. *Antimicrob. Agents Chemother.* **51**, 2954–2964 (2007).
43. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of Staphylococcal Cassette Chromosome *mec* (SCC*mec*): Guidelines for Reporting Novel SCC*mec* Elements. *Antimicrob. Agents Chemother.* **53**, 4961–4967 (2009).
44. Neoh, H., Tan, X.-E., Sapri, H. F. & Tan, T. L. Pulsed-field gel electrophoresis (PFGE): A review of the “gold standard” for bacteria typing and current alternatives. *Infect. Genet. Evol.* **74**, 103935 (2019).
45. Frénay, H. M. E. *et al.* Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**, 60–64 (1996).
46. Schouls, L. M. *et al.* Multiple-Locus Variable Number Tandem Repeat Analysis of *Staphylococcus aureus*: Comparison with Pulsed-Field Gel Electrophoresis and *spa*-Typing. *PLoS One* **4**, e5082 (2009).
47. Schürch, A. C., Arredondo-Alonso, S., Willems, R. J. L. & Goering, R. V. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. *Clin. Microbiol. Infect.* **24**, 350–354 (2018).
48. Lindsay, J. A. Evolution of *Staphylococcus aureus* and MRSA during outbreaks. *Infect. Genet. Evol.* **21**, 548–553 (2014).
49. Aires de Sousa, M. & de Lencastre, H. Evolution of Sporadic Isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Hospitals and Their Similarities to Isolates of Community-Acquired MRSA. *J. Clin. Microbiol.* **41**, 3806–3815 (2003).
50. Johnson, A. P. *et al.* Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). *J. Antimicrob. Chemother.* **48**, 143–144 (2001).
51. Ellington, M. J. *et al.* Decline of EMRSA-16 amongst methicillin-resistant *Staphylococcus aureus* causing bacteraemias in the UK between 2001 and 2007. *J. Antimicrob. Chemother.* **65**, 446–448 (2010).
52. Holden, M. T. G. *et al.* A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. *Genome Res.* **23**, 653–664 (2013).
53. Aires de Sousa, M. & Lencastre, H. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunol. Med. Microbiol.* **40**, 101–111 (2004).
54. Aires-de-Sousa, M., Correia, B. & de Lencastre, H. Changing Patterns in Frequency of Recovery of Five Methicillin-Resistant *Staphylococcus aureus* Clones in Portuguese Hospitals: Surveillance over a 16-Year Period. *J. Clin. Microbiol.* **46**, 2912–2917 (2008).
55. Amorim, M. L. *et al.* Changes in the clonal nature and antibiotic resistance profiles of methicillin-resistant *Staphylococcus aureus* isolates associated with spread of the EMRSA-15 clone in a tertiary care Portuguese hospital. *J. Clin. Microbiol.* **45**, 2881–2888 (2007).
56. Melter, O. *et al.* Methicillin-Resistant *Staphylococcus aureus* Clonal Types in the Czech Republic. *J. Clin. Microbiol.* **37**, 2798–2803 (1999).

57. Melter, O. *et al.* Update on the Major Clonal Types of Methicillin-Resistant *Staphylococcus aureus* in the Czech Republic. *J. Clin. Microbiol.* **41**, 4998–5005 (2003).
58. Melter, O. *et al.* Emergence of EMRSA-15 clone in hospitals throughout the Czech Republic. *Eurosurveillance.* **11**, E060803.6 (2006).
59. Strommenger, B., Cuny, C., Werner, G. & Witte, W. Obvious Lack of Association Between Dynamics of Epidemic Methicillin-Resistant *Staphylococcus aureus* in Central Europe and *agr* Specificity Groups. *Eur. J. Clin. Microbiol. Infect. Dis.* **23**, 15–19 (2004).
60. Conceição, T. *et al.* Replacement of methicillin-resistant *Staphylococcus aureus* clones in Hungary over time: a 10-year surveillance study. *Clin. Microbiol. Infect.* **13**, 971–979 (2007).
61. Chongtrakool, P. *et al.* Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: A proposal for a new nomenclature for SCC*mec* elements. *Antimicrob. Agents Chemother.* **50**, 1001–1012 (2006).
62. Hsu, L.-Y. *et al.* Evolutionary dynamics of methicillin-resistant *Staphylococcus aureus* within a healthcare system. *Genome Biol.* **16**, 81 (2015).
63. Chen, C.-J. *et al.* Molecular Epidemiology and Antimicrobial Resistance of Methicillin-Resistant *Staphylococcus aureus* Bloodstream Isolates in Taiwan, 2010. *PLoS One* **9**, e101184 (2014).
64. McDougal, L. K. *et al.* Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant *Staphylococcus aureus* Isolates from the United States: Establishing a National Database. *J. Clin. Microbiol.* **41**, 5113–5120 (2003).
65. Holden, M. T. G. & Lindsay, J. A. Whole genomes: sequence, microarray, and systems biology. in *Staphylococcus: Molecular Genetics* (ed. Lindsay, J. A. (ed)) 1–28 (Caister Academic Press, 2008).
66. Lindsay, J. A. *et al.* Microarrays Reveal that Each of the Ten Dominant Lineages of *Staphylococcus aureus* Has a Unique Combination of Surface-Associated and Regulatory Genes. *J. Bacteriol.* **188**, 669–676 (2006).
67. McCarthy, A. J. & Lindsay, J. A. 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 (2010).
68. Lindsay, J. A. *Staphylococci: Evolving Genomes.* *Microbiol. Spectr.* **7**, GPP3-0071–2019 (2019).
69. van Wamel, W. J. B., Rooijakkers, S. H. M., Ruyken, M., van Kessel, K. P. M. & van Strijp, J. A. G. The Innate Immune Modulators Staphylococcal Complement Inhibitor and Chemotaxis Inhibitory Protein of *Staphylococcus aureus* Are Located on β -Hemolysin-Converting Bacteriophages. *J. Bacteriol.* **188**, 1310–1315 (2006).
70. McCarthy, A. J., Witney, A. A. & Lindsay, J. A. *Staphylococcus aureus* Temperate Bacteriophage: Carriage and Horizontal Gene Transfer is Lineage Associated. *Front. Cell. Infect. Microbiol.* **2**, 6 (2012).
71. Larsen, J. *et al.* Evidence for Human Adaptation and Foodborne Transmission of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* **63**, 1349–1352 (2016).
72. Grumann, D., Nübel, U. & Bröker, B. M. *Staphylococcus aureus* toxins – Their functions and genetics. *Infect. Genet. Evol.* **21**, 583–592 (2014).
73. Saeed, K. *et al.* Pantón–Valentine leukocidin-positive *Staphylococcus aureus*: a position statement from the International Society of Chemotherapy. *Int. J. Antimicrob. Agents* **51**, 16–25 (2018).
74. Planet, P. J. *et al.* Emergence of the Epidemic Methicillin-Resistant *Staphylococcus aureus* Strain USA300 Coincides with Horizontal Transfer of the Arginine Catabolic Mobile Element and *speG*-mediated Adaptations for Survival on Skin. *MBio* **4**, e00889-13 (2013).

75. Diep, B. A. *et al.* The Arginine Catabolic Mobile Element and Staphylococcal Chromosomal Cassette mec Linkage: Convergence of Virulence and Resistance in the USA300 Clone of Methicillin-Resistant *Staphylococcus aureus*. *J. Infect. Dis.* **197**, 1523–1530 (2008).
76. Thurlow, L. R. *et al.* Functional Modularity of the Arginine Catabolic Mobile Element Contributes to the Success of USA300 Methicillin-Resistant *Staphylococcus aureus*. *Cell Host Microbe* **13**, 100–107 (2013).
77. Li, M. *et al.* MRSA epidemic linked to a quickly spreading colonization and virulence determinant. *Nat. Med.* **18**, 816–819 (2012).
78. Planet, P. J. *et al.* Architecture of a Species: Phylogenomics of *Staphylococcus aureus*. *Trends Microbiol.* **25**, 153–166 (2017).
79. Montgomery, C. P., Boyle-Vavra, S. & Daum, R. S. The arginine catabolic mobile element is not associated with enhanced virulence in experimental invasive disease caused by the community-associated methicillin-resistant *Staphylococcus aureus* USA300 genetic background. *Infect. Immun.* **77**, 2650–2656 (2009).
80. Wu, K., Conly, J., McClure, J.-A., Kurwa, H. A. & Zhang, K. Arginine Catabolic Mobile Element in Evolution and Pathogenicity of the Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strain USA300. *Microorganisms* **8**, 275 (2020).
81. Yamaguchi, T. *et al.* Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. *Mol. Microbiol.* **38**, 694–705 (2000).
82. Yamaguchi, T. *et al.* Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infect. Immun.* **69**, 7760–7771 (2001).
83. Azarian, T. *et al.* Genomic Epidemiology and Global Population Structure of Exfoliative Toxin A-Producing *Staphylococcus aureus* Strains Associated With Staphylococcal Scalded Skin Syndrome. *Front. Microbiol.* **12**, 1–11 (2021).
84. Malachowa, N. & DeLeo, F. R. Mobile genetic elements of *Staphylococcus aureus*. *Cell. Mol. Life Sci.* **67**, 3057–3071 (2010).
85. Liu, J. *et al.* Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant *Staphylococcus aureus*. *Microb. Pathog.* **101**, 56–67 (2016).
86. Foster, T. J. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiol. Rev.* **41**, 430–449 (2017).
87. Baines, S. L. *et al.* Convergent Adaptation in the Dominant Global Hospital Clone ST239 of Methicillin-Resistant *Staphylococcus aureus*. *MBio* **6**, e00080-15 (2015).
88. Knight, G. M. *et al.* Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time. *J. Antimicrob. Chemother.* **67**, 2514–2522 (2012).
89. Chua, K., Laurent, F., Coombs, G., Grayson, M. L. & Howden, B. P. Not Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA)! A Clinician's Guide to Community MRSA - Its Evolving Antimicrobial Resistance and Implications for Therapy. *Clin. Infect. Dis.* **52**, 99–114 (2011).
90. Kadlec, K., Feßler, A. T., Hauschild, T. & Schwarz, S. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Infect.* **18**, 745–755 (2012).
91. Dancer, S. J. Importance of the environment in methicillin-resistant *Staphylococcus aureus* acquisition: the case for hospital cleaning. *Lancet Infect. Dis.* **8**, 101–113 (2008).
92. Boyce, J. M., Potter-Bynoe, G., Chenevert, C. & King, T. Environmental contamination due to methicillin-resistant *Staphylococcus aureus*: possible infection control implications. *Infect. Control Hosp. Epidemiol.* **18**, 622–7 (1997).

93. Bures, S., Fishbain, J. T., Uyehara, C. F. T., Parker, J. M. & Berg, B. W. Computer keyboards and faucet handles as reservoirs of nosocomial pathogens in the intensive care unit. *Am. J. Infect. Control* **28**, 465–471 (2000).
94. Ledwoch, K. *et al.* How dirty is your QWERTY? The risk of healthcare pathogen transmission from computer keyboards. *J. Hosp. Infect.* **112**, 31–36 (2021).
95. Fritz, S. A. *et al.* Contamination of Environmental Surfaces With *Staphylococcus aureus* in Households With Children Infected With Methicillin-Resistant *S. aureus*. *JAMA Pediatr.* **168**, 1030–1038 (2014).
96. Uhlemann, A.-C. *et al.* The Environment as an Unrecognized Reservoir for Community-Associated Methicillin Resistant *Staphylococcus aureus* USA300: A Case-Control Study. *PLoS One* **6**, e22407 (2011).
97. Kramer, A., Schwebke, I. & Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* **6**, 130 (2006).
98. Rountree, P. M. The effect of desiccation on the viability of *Staphylococcus aureus*. *J. Hyg. (Lond.)* **61**, 265–272 (1963).
99. Beard-Pegler, M. A., Stubbs, E. & Vickery, A. M. Observations on the resistance to drying of staphylococcal strains. *J. Med. Microbiol.* **26**, 251–255 (1988).
100. Wagenvoort, J. H. T., Sluijsmans, W. & Penders, R. J. R. Better environmental survival of outbreak vs. sporadic MRSA isolates. *J. Hosp. Infect.* **45**, 231–234 (2000).
101. Baldan, R. *et al.* Epidemic MRSA clone ST22-IV is more resistant to multiple host- and environment-related stresses compared with ST228-I. *J. Antimicrob. Chemother.* **70**, 757–765 (2015).
102. Kluytmans, J., van Belkum, A. & Verbrugh, H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin. Microbiol. Rev.* **10**, 505–520 (1997).
103. von Eiff, C., Becker, K., Machka, K., Stammer, H. & Peters, G. Nasal Carriage as a Source of *Staphylococcus aureus* Bacteremia. *N. Engl. J. Med.* **344**, 11–16 (2001).
104. Wertheim, H. F. L. *et al.* Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* **364**, 703–705 (2004).
105. Kluytmans, J. A. J. W. *et al.* Nasal Carriage Of *Staphylococcus aureus* As A Major Risk Factor For Wound Infections After Cardiac Surgery. *J. Infect. Dis.* **171**, 216–219 (1995).
106. Kalmeijer, M. D., Nieuwland-Bollen, E. van, Bogaers-Hofman, D., Baere, G. A. J. de & Kluytmans, J. A. J. W. Nasal Carriage of *Staphylococcus aureus*: Is a Major Risk Factor for Surgical-Site Infections in Orthopedic Surgery. *Infect. Control Hosp. Epidemiol.* **21**, 319–323 (2000).
107. Muñoz, P. *et al.* Nasal carriage of *S. aureus* increases the risk of surgical site infection after major heart surgery. *J. Hosp. Infect.* **68**, 25–31 (2008).
108. Nouwen, J. L., Fieren, M. W. J. A., Snijders, S., Verbrugh, H. A. & Van Belkum, A. Persistent (not intermittent) nasal carriage of *Staphylococcus aureus* is the determinant of CPD-related infections. *Kidney Int.* **67**, 1084–1092 (2005).
109. Turner, N. A. *et al.* Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat. Rev. Microbiol.* **17**, 203–218 (2019).
110. Nouwen, J., Boelens, H., van Belkum, A. & Verbrugh, H. Human Factor in *Staphylococcus aureus* Nasal Carriage. *Infect. Immun.* **72**, 6685–6688 (2004).
111. van Belkum, A. *et al.* Reclassification of *Staphylococcus aureus* Nasal Carriage Types. *J. Infect. Dis.* **199**, 1820–1826 (2009).

112. Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
113. Liu, C. M. *et al.* *Staphylococcus aureus* and the ecology of the nasal microbiome. *Sci. Adv.* **1**, e1400216 (2015).
114. Yan, M. *et al.* Nasal Microenvironments and Interspecific Interactions Influence Nasal Microbiota Complexity and *S. aureus* Carriage. *Cell Host Microbe* **14**, 631–640 (2013).
115. Krismer, B., Weidenmaier, C., Zipperer, A. & Peschel, A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nat. Rev. Microbiol.* **15**, 675–687 (2017).
116. Iwase, T. *et al.* *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* **465**, 346–349 (2010).
117. Sugimoto, S. *et al.* *Staphylococcus epidermidis* Esp degrades specific proteins associated with *staphylococcus aureus* biofilm formation and host-pathogen interaction. *J. Bacteriol.* **195**, 1645–1655 (2013).
118. Zipperer, A. *et al.* Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* **535**, 511–516 (2016).
119. Selva, L. *et al.* Killing niche competitors by remote-control bacteriophage induction. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 1234–1238 (2009).
120. Uehara, Y. *et al.* H₂O₂ produced by viridans group streptococci may contribute to inhibition of methicillin-resistant *Staphylococcus aureus* colonization of oral cavities in newborns. *Clin. Infect. Dis.* **32**, 1408–1413 (2001).
121. Bode, L. G. M. *et al.* Preventing Surgical-Site Infections in Nasal Carriers of *Staphylococcus aureus*. *N. Engl. J. Med.* **362**, 9–17 (2010).
122. World Health Organization. Evidence-Based Recommendations on Measures for the Prevention of Surgical Site Infection. in *Global Guidelines for the Prevention of Surgical Site Infection* (2018). Available from: <https://www.ncbi.nlm.nih.gov/books/NBK536431/>
123. Septimus, E. J. & Schweizer, M. L. Decolonization in prevention of health care-associated infections. *Clin. Microbiol. Rev.* **29**, 201–221 (2016).
124. Kwiecien, K. *et al.* Architecture of antimicrobial skin defense. *Cytokine Growth Factor Rev.* **49**, 70–84 (2019).
125. Peacock, S. J., de Silva, I. & Lowy, F. D. What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiol.* **9**, 605–610 (2001).
126. Bitschar, K., Wolz, C., Krismer, B., Peschel, A. & Schitteck, B. Keratinocytes as sensors and central players in the immune defense against *Staphylococcus aureus* in the skin. *J. Dermatol. Sci.* **87**, 215–220 (2017).
127. Hashimoto, M. *et al.* Lipoprotein is a predominant Toll-like receptor 2 ligand in *Staphylococcus aureus* cell wall components. *Int. Immunol.* **18**, 355–362 (2006).
128. Nguyen, M. T. & Götz, F. Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence. *Microbiol. Mol. Biol. Rev.* **80**, 891–903 (2016).
129. Miller, L. S. & Cho, J. S. Immunity against *Staphylococcus aureus* cutaneous infections. *Nat. Rev. Immunol.* **11**, 505–518 (2011).
130. Scudiero, O. *et al.* Human Defensins: A Novel Approach in the Fight against Skin Colonizing *Staphylococcus aureus*. *Antibiotics* **9**, 198 (2020).
131. Bernard, J. J. & Gallo, R. L. Protecting the boundary: the sentinel role of host defense peptides in the skin. *Cell. Mol. Life Sci.* **68**, 2189–2199 (2011).

132. Braff, M. H., Zaiou, M., Fierer, J., Nizet, V. & Gallo, R. L. Keratinocyte Production of Cathelicidin Provides Direct Activity against Bacterial Skin Pathogens. *Infect. Immun.* **73**, 6771–6781 (2005).
133. Kisich, K. O. *et al.* The Constitutive Capacity of Human Keratinocytes to Kill *Staphylococcus aureus* Is Dependent on β -Defensin 3. *J. Invest. Dermatol.* **127**, 2368–2380 (2007).
134. Wanke, I. *et al.* Skin Commensals Amplify the Innate Immune Response to Pathogens by Activation of Distinct Signaling Pathways. *J. Invest. Dermatol.* **131**, 382–390 (2011).
135. Cole, A. L. *et al.* Host innate inflammatory factors and staphylococcal protein A influence the duration of human *Staphylococcus aureus* nasal carriage. *Mucosal Immunol.* **9**, 1537–1548 (2016).
136. Nurjadi, D., Herrmann, E., Hinderberger, I. & Zanger, P. Impaired β -Defensin Expression in Human Skin Links DEFB1 Promoter Polymorphisms With Persistent *Staphylococcus aureus* Nasal Carriage. *J. Infect. Dis.* **207**, 666–674 (2013).
137. Mulcahy, M. E. & McLoughlin, R. M. Host–Bacterial Crosstalk Determines *Staphylococcus aureus* Nasal Colonization. *Trends Microbiol.* **24**, 872–886 (2016).
138. Kalenic, S. *et al.* Comparison of recommendations in national/regional Guidelines for prevention and control of MRSA in thirteen European countries. *Int. J. Infect. Control* **6**, 2 (2010).
139. Duerden, B., Fry, C., Johnson, A. P. & Wilcox, M. H. The Control of Methicillin-Resistant *Staphylococcus aureus* Blood Stream Infections in England. *Open Forum Infect. Dis.* **2**, ofv035 (2015).
140. Carlet, J. *et al.* French National Program for Prevention of Healthcare-Associated Infections and Antimicrobial Resistance, 1992–2008: Positive Trends, But Perseverance Needed. *Infect. Control Hosp. Epidemiol.* **30**, 737–745 (2009).
141. Pan, A. *et al.* A survey of methicillin-resistant *Staphylococcus aureus* control strategies in Italy. *Infection* **41**, 783–789 (2013).
142. World Health Organization. *Antimicrobial resistance: global report on surveillance*. (World Health Organization, 2014). Available from: <https://apps.who.int/iris/handle/10665/112642>
143. Price, L. B. *et al.* *Staphylococcus aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. *MBio* **3**, e00305-11 (2012).
144. Harkins, C. P. *et al.* Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biol.* **18**, 130 (2017).
145. SWAB. *Nethmap 2016. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2015*. <https://swab.nl/nl/nethmap> (2016).
146. SWAB. *Nethmap 2021. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2020*. <https://swab.nl/nl/nethmap> (2021).
147. Reuter, S. *et al.* Building a genomic framework for prospective MRSA surveillance in the United Kingdom and the Republic of Ireland. *Genome Res.* **26**, 263–270 (2016).
148. Dauwalder, O. *et al.* Epidemiology of Invasive Methicillin-Resistant *Staphylococcus aureus* Clones Collected in France in 2006 and 2007. *J. Clin. Microbiol.* **46**, 3454–3458 (2008).

Chapter 2

Markers of epidemiological success of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in European populations

Valérie O. Baede[#], Arya Gupta[#], Gwenan M. Knight[#],
Leo M. Schouls, Ken Laing, Mehri Tavakol, Anaïs Barray,
Sake J. de Vlas, Anneke S. de Vos, Antoni P.A. Hendrickx,
Madeeha Khan, Mirjam E. Kretzschmar, Willem J.B. van Wamel,
Gérard Lina, François Vandenesch, Margreet C. Vos,
Adam A. Witney^{*}, Jean-Philippe Rasigade^{*}, Jodi A. Lindsay^{*}

^{#,*} These authors contributed equally

Submitted

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections impose a considerable burden on health systems, yet there is remarkable variation in the global incidence and epidemiology of MRSA. The MACOTRA consortium aimed to identify bacterial markers of epidemic success of MRSA isolates in Europe using a representative MRSA collection originating from France, the Netherlands and the United Kingdom.

Operational definitions of success were defined in consortium meetings to compose a balanced strain collection of successful and sporadic MRSA isolates. Isolates were subjected to antimicrobial susceptibility testing and whole genome sequencing. Resistance genes were identified and phylogenetic trees constructed. Markers of epidemiological success were identified using genome-based time-scaled haplotypic density (THD) analysis and linear regression. Antimicrobial usage data from ESAC-Net was compared to national MRSA incidence data.

Heterogeneity of MRSA isolate collections across countries hampered the use of a unified operational definition of success, so country-specific approaches were used to establish the MACOTRA strain collection. Phenotypic antimicrobial resistance varied within related MRSA populations and across countries. In THD analysis, fluoroquinolone, macrolide and mupirocin resistance were associated with MRSA success, while gentamicin, rifampicin and trimethoprim resistance were associated with sporadicity. Usage of beta-lactams, fluoroquinolones and macrolides across 29 European countries correlated with MRSA incidence.

Our results are the strongest yet to associate MRSA antibiotic resistance profiles to incidence of infection and successful clonal spread, which varied by country. Usage of the same antimicrobials correlated with MRSA incidence across Europe. Harmonized isolate collection, typing, resistance profiling and alignment with antimicrobial usage over time will aid comparisons and further support country-specific interventions to reduce MRSA burden.

Introduction

Antimicrobial resistance (AMR) is considered to be the greatest threat to the future of modern medicine, and MRSA is estimated to be the most common cause of AMR associated deaths globally and second in Europe ^{1,2}. MRSA carriage is a risk factor for subsequent infection ³⁻⁵. MRSA are resistant to virtually the full spectrum of beta-lactam antimicrobials due to the *mecA* carrying *SCCmec* mobile element acquired on multiple occasions into different *S. aureus* genetic lineages, resulting in a range of different MRSA clones. Approximately a dozen clones, defined by their lineage (also called clonal cluster (CC)) and associated *SCCmec* cassette type dominate the MRSA population globally. The dominant clone may differ in different geographic settings, and may even change over time ^{4,6,7}. Resistance to almost all classes of antimicrobials can be found in MRSA, although fully drug-resistant isolates are rare. Furthermore, the incidence of MRSA infection can vary widely between countries, but the reasons for these geographical differences are poorly understood.

Each country has developed distinct strategies for collecting, testing, typing and reporting of MRSA isolates ⁸. This variation in approaches obstructs comparisons and proper aggregation of data and therefore thorough epidemiological analysis on an international level and could potentially hide or over-estimate common markers of successful clones across geographic settings. The implementation of whole genome sequencing analysis can aid international data comparison and can be used to search for genotypic markers of successful isolates.

The UK, France and the Netherlands represent countries with very differing incidences of MRSA infection and their spread are dominated by different clones ⁹⁻¹¹. Here we used two approaches to identify epidemiologically successful isolates in each country and searched for genetic and AMR biomarkers of success. Further analysis of resistance markers revealed associations between the usage of specific antimicrobial classes and MRSA incidence across 29 European countries, identifying potential targets for stewardship interventions.

Methods

Operational definition and collection of successful and sporadic (unsuccessful) isolates

Collaborators from each country, France, the Netherlands and the UK, identified their country-specific epidemiological characteristics of MRSA success over time, which included incidence of infection and identification of dominant clones using local methodology. This approach relied on published and reference centre resources, as well as investigation of local available strain collections using local typing methods. Using this information, the most useful method of identifying successful and sporadic (unsuccessful) isolates within collections was identified and described, and the criteria for identifying individual examples for strain analysis proposed. Where common criteria across countries could be identified this was used.

Whole genome sequencing and epidemiological clustering

Whole genome sequencing of isolates was generated at St. George's University of London, UK (SGUL) using the Illumina MiSeq platform. UK isolates from 2003, 2006, 2008 and 2009 (n=168) had been previously sequenced ¹². DNA

was extracted using the PurElute (Edge Biosystems) kit and 2.5 μ l of lysostaphin (Sigma Aldrich). Sequence reads were aligned to reference genomes (RefSeq NC_002952, NC_017763, NC_002745) using bwa mem 0.7.17-r1188 and sites called with bcftools mpileup (v1.9) ¹³. Sites were filtered based on the following criteria: mapping quality (MQ) above 30, site quality score (QUAL) above 30, at least 4 reads covering each site with at least 2 reads mapping to each strand, at least 75% of reads supporting site (DP4) Sites which failed these criteria in any isolate were removed from the analysis. Phylogenetic reconstruction was performed using RAxML v8.2.3 with a GTR model of nucleotide substitution and a GAMMA model of rate heterogeneity, branch support values were determined using 1000 bootstrap replicates ¹⁴. Genomes were also assembled using Shovill v1.0.9 and resistance genes identified using Abricate and the CARD database, virulence genes identified using the VFDB database ^{15,16}. Genes associated with operational success were compared using Chi-squared tests as 5% significance threshold.

Genome-based estimation of epidemic success

Assembled genomes were annotated using prokka ¹⁷ and the pangenome assessed using roary ¹⁸ using default parameters. Genes associated with success and resistance phenotypes were determined using pyseer ¹⁹. First unitigs were counted with unitig-counter ²⁰ and unitigs with significant associations with the phenotypes determined using a linear mixed model, correcting for population structure using a kinship matrix generated by an all isolate phylogenetic tree, generated as described above.

Antimicrobial susceptibility testing

EUCAST disk diffusion methodology ²¹ was used to test for sensitivity to 14 antibiotic disks (Oxoid, Basingstoke, UK): cefoxitin (30 μ g), ampicillin (2 μ g), chloramphenicol (10 μ g), ciprofloxacin (5 μ g), clindamycin (2 μ g), erythromycin (15 μ g), gentamicin (10 μ g), tobramycin (10 μ g), fusidic acid (10 μ g), linezolid (10 μ g), mupirocin (200 μ g), rifampicin (5 μ g), tetracycline (30 μ g), and trimethoprim (5 μ g). Ninety-five of the UK isolates were previously tested using BSAC criteria ^{9,12}. Following determination of antimicrobial susceptibility of the MRSA isolate collection, the distribution of phenotypic resistances and successful isolates in each country was compared using Chi-squared tests, $p < 0.05$; if any expected values were < 5 , Fisher's exact test was used.

Time-scaled haplodeficiency (THD) analysis

Phyloepidemiological methods leverage genome sequences to derive epidemiological quantities including epidemic success. We used the time-scaled haplotypic density method to examine the factors predicting epidemic success of MRSA ²²⁻²⁴. THD assigned relative indices of epidemic success, over a defined time period, to each isolate in the dataset, on the basis of the branching density and distribution of genetic distances separating it from other isolates.

Genetic distances were defined as the pairwise number of SNPs between isolates, based on sequence alignment with respect to the NC017763 MRSA reference genome. THD was computed from the matrix of pairwise SNP distances, using an effective genome size of 3×10^6 bp and an evolutionary rate of 10^{-6} substitution per site per year as previously determined for *S. aureus* ²⁵. An epidemic period of 5 years was used as the THD timescale to restrict analysis to short-term success.

Four isolates (MAC209, 226, 204, and 188) exhibited THD indices $<1e-8$ and were excluded. THD analyses were also restricted to isolates belonging to CCs with a sample size >10 , namely CC1, CC22, CC30, CC45, CC5, CC8, CC80 and CC398, to avoid including outliers in the models. $N=368$ isolates were included in the final THD analyses.

Potential predictors of success, such as antimicrobial drug resistance patterns, were identified using linear regression models with THD indices as the response variable. Adjustment for potential confounders was conducted, where indicated in text, by including confounders as model co-variables or as random effects, as appropriate. Associations of predictors with THD indices are reported as regression slope coefficients with 95% confidence intervals computed using likelihood profiling. All analyses used R software version 4.0.2 with additional packages *thd* (<https://github.com/rasigadelab/thd>) and *lmerTest*^{26–28}.

AMR and antimicrobial usage

The sum of estimated incidence of all infection types caused by MRSA was used to give an overall MRSA annual incidence per 100 000 population data across 29 European countries². This was based on EARS-Net data adjusted for coverage and usage of diagnostics for the year 2015². Antimicrobial consumption data for 2015 was from ESAC-Net (ecdc.europa.eu) and expressed as defined daily dose (DDD) per 1000 inhabitants per day. The data for some countries was split into community and healthcare usage and sourced from national sales and reimbursement data. We tested both linear (shown here) and exponential trend associations (in Supplementary data), with an F test for significance. All data management and analyses are provided in the Supplementary data and as code in a Github repository: https://github.com/gwenknight/mrsa_inf_abx. Regression models were fitted using the “lm()” function in R²⁷.

Results

Operational definition of epidemiologically successful and sporadic isolates

Details of isolate selection

Country-specific strategies were adopted with different inclusion criteria for storage and surveillance. Detailed information for each strategy is given below. Full details of all collected isolates are in Supplementary Table S1.

France. Representative isolates from France were selected from the collection of the French *National* Reference Centre for Staphylococcus (NRC), Lyon, France. This collection consists of methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA isolates referred to the NRC by approximately 380 laboratories for microbiological expertise and does not include isolates from clinical studies or cohorts. From 2014 onwards, all isolates in the collection have been subjected to DNA array profiling using 332-loci Alere Staphytype (Alere Technologies GmbH, Jena, Germany) as described elsewhere²⁹. Isolates are assigned to multilocus sequence types (STs) and clonal complexes (CCs), as well as specific lineages such as ST8 USA300³⁰, by comparing whole-array hybridization profiles to previously MLST-typed reference strains in a dedicated database³¹.

Isolates with a ST8 USA300 profile were readily classified as sporadic in France based on their limited local spread compared to other countries, in spite of

repeated introductions²⁵. Other isolates were classified as successful or sporadic and stratified across major CCs using the following rationale. Major CCs were defined as those with >20 MRSA isolates in the 2014-17 collection (which had a total of 5,457 isolates including 1,382 MRSA). Eight major CCs were found, namely CC8 (n=216), CC5 (n=200), CC80 (n=171), CC30 (n=39), CC22 (n= 36), CC1 (n=33), CC88 (n = 27) and CC59 (n=25), totalling 747 isolates.

The successful or sporadic classification of isolates was then based on their subtype cluster frequency within each major CC. This rationale for clustering was to ensure that the intra-cluster variability within a CC was constant. First, microarray data were subjected to hierarchical clustering using Ward's method to produce one dendrogram per CC³². Clusters of isolate subtypes were found in each dendrogram using equal-height tree cutting, where the number of clusters was arbitrarily defined as one-fifth of the number of isolates in the CC, up to a maximum of 10 clusters. This method ensured consistent subtyping of isolates across CCs of varying size. Subtype clusters in each CC were sorted by size. The largest clusters totalling >25% of CC size were labelled as 'successful' while the smallest clusters totalling >25% of CC size were labelled as 'sporadic'. Other subtype clusters were considered inconclusive and excluded.

The above classification criteria resulted in 316 isolates classified as 'successful' (CC8 = 97, CC5 = 81, CC80 = 67, CC30 = 21, CC22 = 11, CC1 = 13, CC88 = 18 and CC59 = 8) and 152 isolates as 'sporadic' (CC8 = 40, CC5 = 37, CC80 = 42, CC30 = 9, CC22 = 8, CC1 = 4, CC88 = 6 and CC59 = 6). A final subset of 96 isolates were selected using balanced sampling across CCs, as well as between successful and sporadic isolates within each CC.

Netherlands. *Type-Ned MRSA database* - As surveillance and collection of MRSA isolates along with relevant epidemiological data is mandatory in the Netherlands, the Dutch National Institute for Public Health and the Environment (Rijksinstituut voor volksgezondheid en milieu [RIVM]) has been receiving and storing MRSA isolates collected through the national surveillance system. This system includes all Dutch Medical Microbiological Laboratories (MML) associated with general practitioners, regional and university hospitals, long term care facilities, and laboratories in Dutch territories overseas. One isolate per person per year is included. These include clinical isolates as well as colonisation isolates, irrespective of the reason for detection, either by contact search, increased risk factors (see below) or clinical samples. When both colonisation and clinical isolates are available, a clinical isolate is preferred, but in practice the first isolated MRSA from a person will be included. All data is collected in the Type-Ned MRSA database. This includes MML of submission, all relevant personal data and epidemiological data, such as gender, age and sample site. Patient privacy is guaranteed under the Dutch law.

Following search and destroy (S&D), a policy implemented in the Netherlands since 1988, every patient at risk for MRSA colonisation is screened at hospital or nursing home admission and placed in pre-emptive isolation awaiting culture results. Subsequently, patients with MRSA positive culture are kept in strict isolation during their hospital stay and offered a treatment to eliminate colonisation, preferably and mostly after discharge and being recovered. Before treatment, household members are tested on carriage and thus transmission and offered an elimination treatment together and at the same moment with the index carrier when positive. Risks for MRSA colonisation were defined by the former Dutch Working party for Infection Prevention (WIP; 1981-2017) and include, among

others, contact with an MRSA carrier, recent stay in a hospital abroad and contact with farmed pigs, veal calves or broilers³³. The assumed origin of MRSA acquisition is classified by infection control practitioners, based on the WIP risk categories, and reported in the Type-Ned database. Occasionally, MRSA is isolated from patients not targeted by S&D, for example in a clinical sample (MRSA of unknown origin; MUO^{34,35}). These findings result in contact tracing which aims to screen all exposed contacts to detect and prevent MRSA outbreaks. Sometimes, this results in identifying a MRSA isolate of different genetic origin than the original MRSA isolate for which the contact search was initiated. These isolates are defined as unexpected findings which start new contact tracings. When no transmission of these unexpected MRSA types is found and their prevalence in the Netherlands is low, we define these MRSA types as unsuccessful (sporadic), as these were carried by hospitalised patients without any contact precautions, and did not show transmission, where another MRSA type in that particular hospital setting had spread. These in particular identified isolates were included as unsuccessful isolates NL4 (see below).

Strain selection

The period 2008-2017 was chosen to ensure overlap in time with the selection period of British and French isolates. During 2008-2017, ± 32.000 MRSA isolates were collected through national surveillance. Aside from livestock-associated (LA) MRSA clade MC0398, the following MLVA -Complexes (MCs) were most prevalent: MC0005, MC0008, MC0022, MC0045, MC0030 and MC0001 (Table 1). As the latter six MC corresponded with frequently found MLST-CC in the UK and France, a subset of isolates belonging to these MC were selected for MACOTRA. To narrow our search and account for changes in prevalence over time, we chose to select isolates from sampling years 2008 and 2017 only. We aimed to select 12 isolates per all six MCs consisting of six isolates for each sampling year per MC. During selection, isolates originating from as many different MMLs as possible were chosen. If a further choice was possible, the earliest submitted isolates were preferred. Four independent selection methods (described below and depicted in Figure 1) were used to complete the collection Dutch isolates.

	Prevalence per sampling year										
MLVA-Complex	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Total
MC0398	41	42	40	40	38	34	30	28	25	25	34
MC0005	15	14	15	15	15	14	18	13	16	13	15
MC0008	15	16	14	14	13	16	14	12	12	12	14
MC0022	5	5	8	5	6	7	8	12	9	12	8
MC0045	8	6	5	8	8	8	11	10	7	6	8
MC0030	3	4	4	4	5	5	4	5	6	6	5
MC0001	1	2	2	1	2	2	3	4	5	5	3
Other MCs	12	12	12	12	14	12	13	16	20	20	15

Table 1. Relative prevalence (%) of included MC in the Netherlands per sampling year

Selection of successful isolates

Individual minimum spanning trees (MST) based on MLVA-types were made for MC0005, MC0008, MC0022, MC0030, MC0045 and MC0001. These MCs are representative of CC5, CC8, CC22, CC30, CC45 and CC1, respectively (expert opinion, Leo M. Schouls). Subsequently, the most prevalent MLVA types were chosen within each MC. From these MLVA types, approximately 8 isolates were selected at random and these were categorized as successful MRSA as they have been able to persist and spread throughout the study period. MC0001 was considered least successful of the six selected prevalent MCs, hence, only 4 isolates from the most prevalent MLVA types were included as successful isolates. This selection method was named NL1. As LA-MRSA, MC0398 is the most prevalent MC in the Netherlands, a separate MST of MC0398 was used to expand the set of the above successful isolates. Three isolates from each sampling year for the most prevalent MLVA types within MC0398 were selected. This selection method was defined as NL2.

Selection of sporadic isolates

For selecting sporadic isolates, four isolates were selected from rare MLVA types within each MC specific MST used in method NL1. These isolates were categorized as sporadic MRSA. For MC0001, 8 isolates from rare MLVA types were included. The collection was expanded with another six isolates from globally dominant clones, which are not prevalent in the Netherlands. Based on epidemiological data, these isolates were categorized as unsuccessful in the Netherlands, as these clones were unable to cause outbreaks in a hospital setting despite repeated introduction (selection method NL3). The added isolates included a pair of ST239 (MC0008) isolates, a pair of USA300 (defined as PVL+ and *spa* type t008) isolates and a pair of MC0080 isolates, with one isolate from 2008 and another from 2017 for each pair.

The set of sporadic isolates was further expanded with six isolates originating from unexpected findings during contact tracing of MRSA outbreaks in Erasmus MC hospital between 2008 and 2017 (selection method NL4). As described above, these MRSA had the chance to spread in a hospital setting but did not show any transmission i.e., were unsuccessful. Furthermore, the MLVA types of these last 6 isolates were present less than 5 times in the Dutch Type-Ned MRSA database between 2008 and 2017.

Additional outbreak isolates

Next-generation sequencing (NGS) of MRSA has been implemented at RIVM since 2017, enabling outbreak investigations based on whole genome MLST (wgMLST). For this approach, 2567 loci of the core and accessory genome were included, and importantly grouping based on wgMLST agreed between NGS groups and MLVA complexes³⁶. The average allelic distance between NGS groups was 1673 alleles, ranging between 1169 and 1959 alleles. Genetic clusters representing possible outbreak clusters were defined as isolates within a NGS group separated by a maximum of 15 genes. In total, 20 isolates were included from five different genetic clusters (range 1- 12 alleles). From each selected genetic cluster, two isolates were defined as successful. For each genetic cluster two genetically closely related, but outside of the genetic cluster (range 43-288 alleles) were selected as sporadic counterparts.

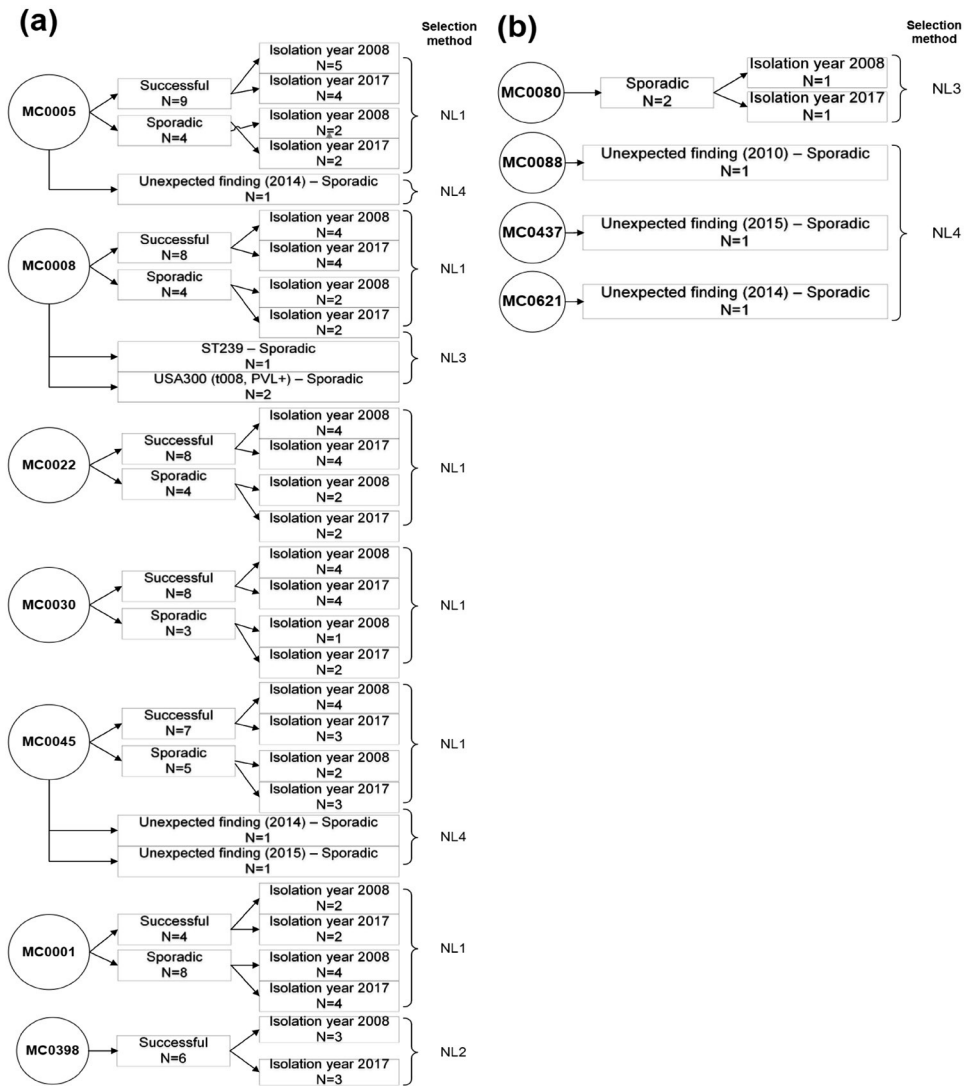
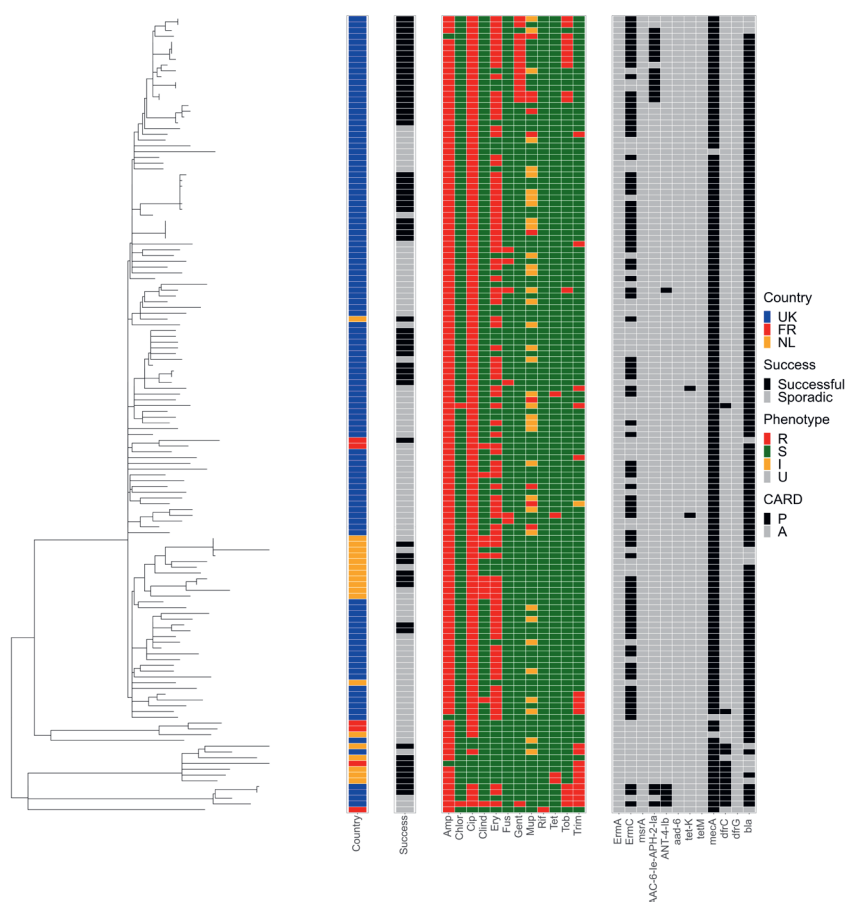


Figure 1. Selection procedure for Dutch isolates.

(a) illustrates the selection procedure of successful and sporadic from the most prevalent MLVA-MC found in the Netherlands; includes LA-MRSA clade, MC0398; (b) describes the selection of sporadic isolates from less prevalent MLVA-MC identified in the Netherlands. NL1: based on selection of prevalent and rare MLVA types of 6 prevalent MLVA-MCs; NL2: selection of prevalent MLVA types of MC0398; NL3: selection based on global successful clones; NL4: selection based on unexpected findings in contact tracings. Unexpected findings imply MRSA of unknown origin, no transmission detected in contact tracing, prevalence <0.025% in MRSA Type-Ned database.

In total, 109 isolates were included in the Dutch part of the MACOTRA strain collection.

United Kingdom. For isolates selection, a collection of well characterised isolates from a single London hospital was utilized. These isolates were representative of the region, and were collected both before and after the reduction in incidence of MRSA infection in the UK in 2007 ¹¹. These St George's University Hospital Trust, London isolates were collected from 1999 – 2009 from a range of specimens sent to the diagnostic microbiology laboratory in a large acute teaching hospital servicing south-west London. In 1999 and 2003, the dominant clone was CC30, interrupted by the emergence and decline of the ST239 clone, and by 2006 were dominated by CC22. Most isolates were resistant to ciprofloxacin and erythromycin. Additionally, resistance to aminoglycoside, trimethoprim, fusidic acid and tetracycline were seen. All isolates had been subjected to WGS ¹ and lineages were confirmed. The collection was supplemented with all stored blood culture MRSA isolates collected at St George's between 2013-2016, where CC22 remained the dominant clone.



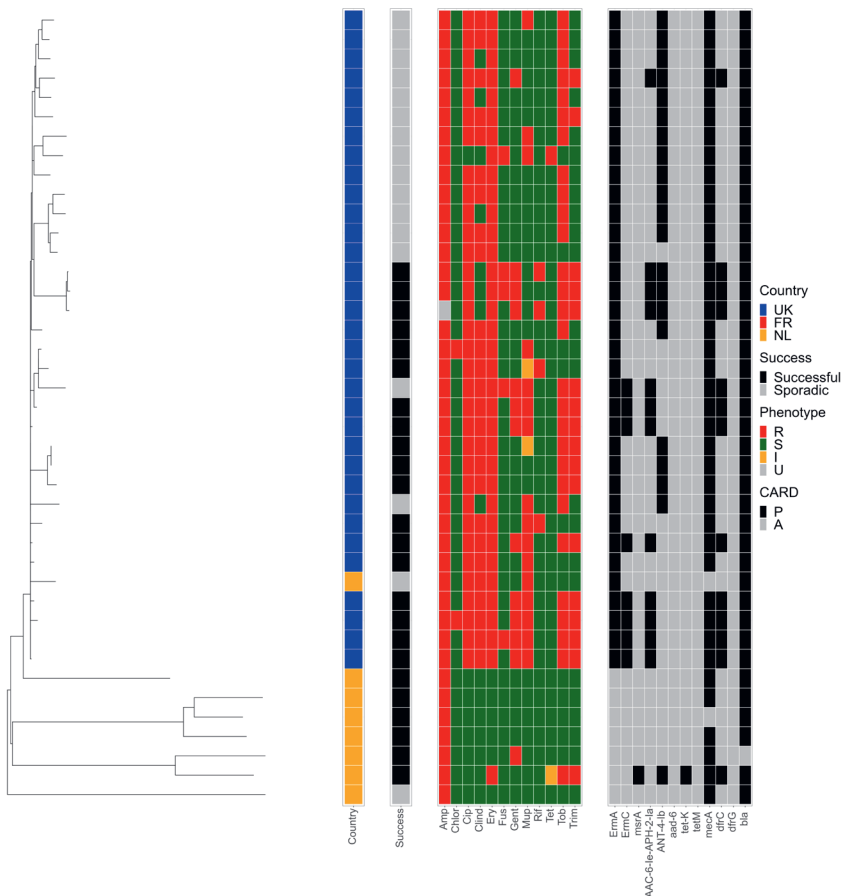


Figure 3. Phylogenetic tree of the CC30 isolates.

Isolates are predominantly from the UK, but have been identified in the Netherlands, however these isolates have not spread. In the Netherlands an unrelated subgroup of diverse CC30s are successful, they are susceptible to ciprofloxacin, erythromycin and aminoglycosides. AMR profiles are highly variable in this CC.

For the total 173 isolates, all those belonging to CC1, CC5, CC8, CC45, ST239, CC51, CC59 ($n=29$, 16.8% of the collection) were classified as sporadic owing to their relatively rare occurrence. For CC22 and CC30 isolates, phylogenetic trees of the collections from all three countries were constructed (Figures 2 and 3), and we defined 'successful' as those UK isolates that belonged to a cluster of two or more isolates on the tree with a SNP difference of <15 bp. The collection assigned 61 successful and 112 sporadic isolates, only 82 isolates were used in the final analysis.

Phylogenetically related isolates were identified across the three countries but differed in dominance and success

Sequence data were submitted to the European Nucleotide Archive (EBI ENA) database with accession number PRJEB47238. Phylogenetic trees of 157 successful and 221 sporadic MRSA clearly identified the different CCs (Figure 4). Each of the three countries was dominated by isolates from different CCs. Clusters within

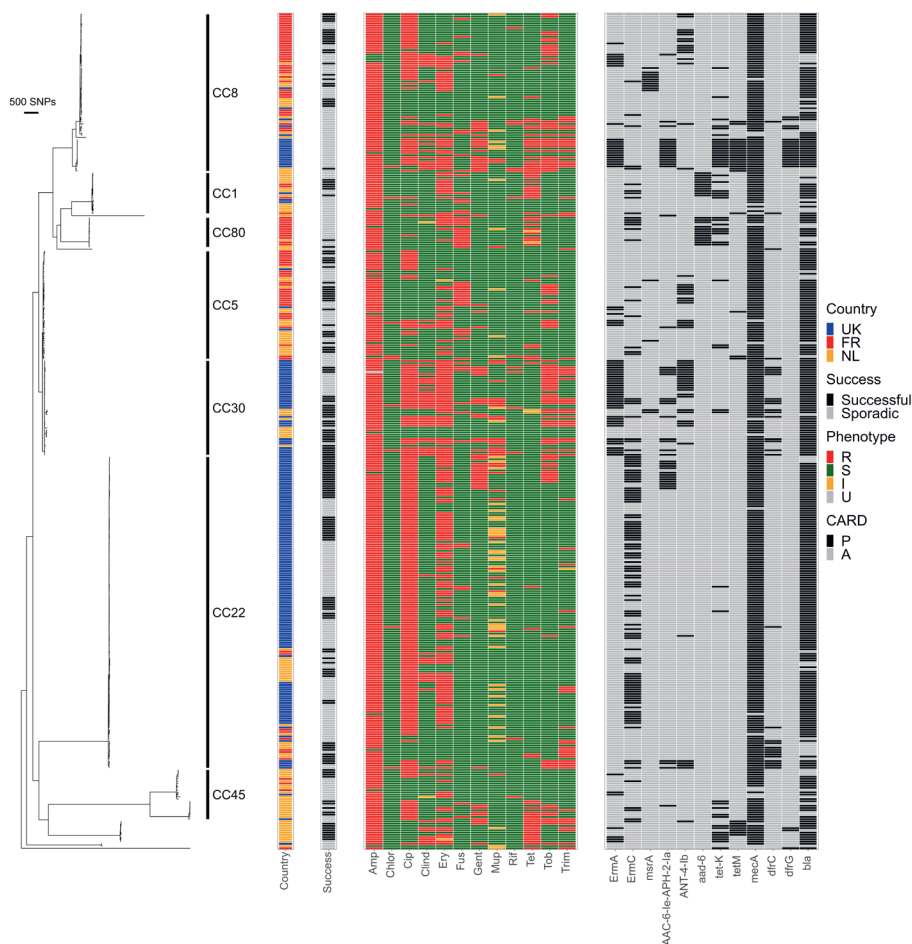


Figure 4. Phylogenetic tree of the collection showing CCs, country and operational success.

Two panels aligned to the tree show the Resistant (R), Susceptible (S), Intermediate (I) and Unknown (U) status of each drug for each isolate, and the Presence (P) and Absence (A) of known resistance genes from the CARD database. Further details of the isolates are in Supplementary Table S1.

sub-branches of each lineage were isolated demonstrating country-specific travel and opportunities of introduction into other countries (Figure 4). Further analysis of CC22 and CC30, the most prevalent CCs, demonstrated that transmission of an isolate to another country did not generally lead to successful localised clusters (Figures 2 and 3).

AMR profiles were highly variable and associated with country

AMR phenotypes and genotypes varied substantially within CCs and across CCs (Figure 4), as well as between countries and operational definitions of success (Figure 5). France had a higher proportion of fusidic acid resistance and a lower proportion of gentamicin, mupirocin and trimethoprim resistance. The Netherlands

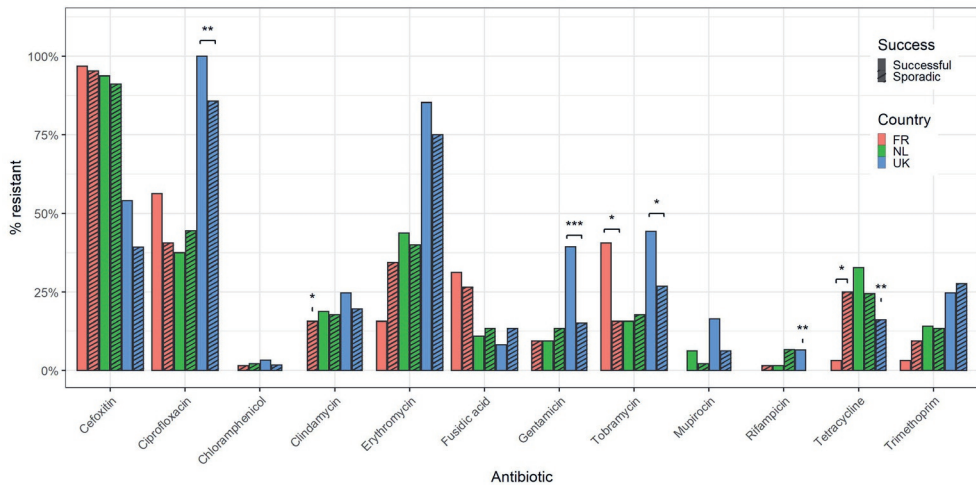


Figure 5. AMR resistance varied between successful and sporadic isolates within countries.

AMR association is marked as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ by χ^2 test.

had a higher proportion of tetracycline resistance and a lower proportion of tobramycin resistance. The UK had a higher proportion of fluoroquinolone, erythromycin, gentamicin, mupirocin, tobramycin and trimethoprim resistance and a lower proportion of tetracycline resistance (Supplementary Figure S4).

Within countries, success in France was associated with tobramycin resistance and sporadic isolates with clindamycin and tetracycline resistance. Success in the UK was associated with ciprofloxacin, gentamicin, rifampicin and tobramycin resistance and sporadic isolates with tetracycline resistance (Figure 5). Across the collection, successful isolates were associated with tobramycin resistance (Supplementary Table S3).

Time-scaled haplotypic density (THD) analysis

THD indices were assigned by genetic distance to other isolates in the collection (Supplementary Table S1) reflecting the rate of transmission and selection over time^{22–24}. THD indices were higher in the UK, suggesting that successful isolates were closely related in this country (Figure 4, Table S1). CC22 was the most successful clone overall (Figure 6b) and the dominant clone in the UK (Figure 6b).

The THD and operational definitions of success did not differ significantly between successful and sporadic isolates (2-sided Mann Whitney test, $p = 0.42$). However, after taking into account the country and CC of each strain as random effects in a mixed-effect linear regression, operational success predicted slightly higher THD values (17.5% increase, 95% CI, 6.7 to 29.4%, $p = 0.001$). These findings indicate that the operational definitions of success failed to capture the epidemic success of a given isolate among the global MRSA population, most likely due to the country-specific definitions; however, operational definitions correctly predicted epidemic success within the same lineage and country.

Then, we leveraged THD indices to examine AMR with epidemic success. We constructed univariate and multivariable models of the THD success index as a function of the inhibition zone diameters for 7 antimicrobial drugs. All models

were adjusted for variations across countries and CCs using random intercepts. In multi-variable analysis, the THD success index correlated positively with ciprofloxacin, erythromycin and mupirocin, and negatively with gentamicin, rifampicin and trimethoprim (Figure 6c).

Genome-wide association study (GWAS) using pyseer to identify mutations and account for lineage variation did not reveal any markers of success. Pyseer correctly identified the most common mutations associated with phenotypic resistance (Supplementary Figure S5). A search for known virulence genes using VFDB identified *tst* (toxic shock syndrome toxin) although prevalence was low (Supplementary Table S1).

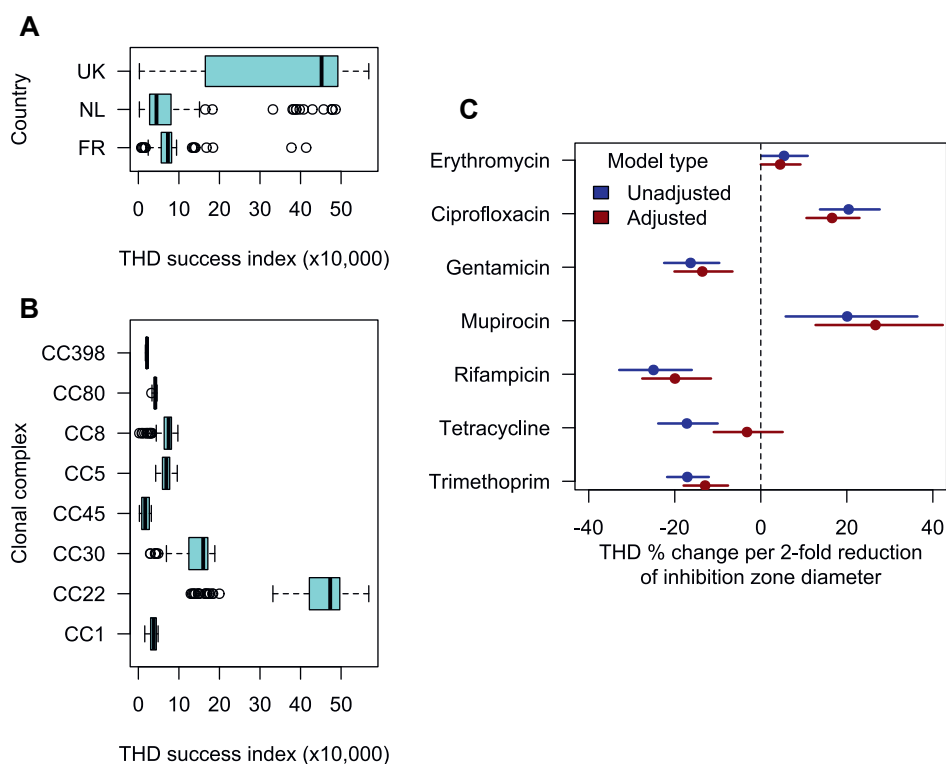


Figure 6. Genome-based analysis of markers of epidemiological success.

Shown are the distributions of THD success indices across countries (A) and clonal complexes (B). Panel C shows pointwise estimates (dots) and 95% confidence intervals (bars) of the coefficients of regression models predicting THD indices with antimicrobial resistance, expressed as units of 2-fold reduction of inhibition zone diameters. Models were either unadjusted (blue bars) or adjusted (multi-variable, red bars). In multivariable regression, THD success indices were predicted by higher ciprofloxacin, erythromycin and mupirocin resistance, and by susceptibility to gentamicin, rifampicin and trimethoprim.

AMR incidence and antimicrobial usage

MRSA incidence of infection varied widely between countries across Europe, ranging from an estimated 1.47 cases in the Netherlands to 102 cases in Portugal per 100,000 persons per year (Figure 7, Supplementary data). Similarly, antimicrobial usage is markedly different across countries. We compared antimicrobial usage with MRSA incidence across 29 European countries to further explore MRSA selection by antibiotics.

MRSA incidence correlated with total beta-lactam usage in 29 countries (Figure 8a, Supplementary data). Specific associations were found between combinations of penicillins, including beta -lactamase inhibitors in the community and hospitals, and 3rd generation cephalosporins in the community. Countries with higher use of beta -lactamase sensitive penicillins in the community had lower MRSA incidence.

We also identified a correlation between MRSA incidence and fluoroquinolone usage (Figure 8b). We note that Portugal is an outlier in our data with very high MRSA incidence. When excluding Portugal from analysis, additional correlations were found between MRSA incidence with macrolide use in the community and aminoglycoside use in hospitals (Figure 8b). Correlations between MRSA incidence and other classes of antimicrobials were not significant (Supplementary data).

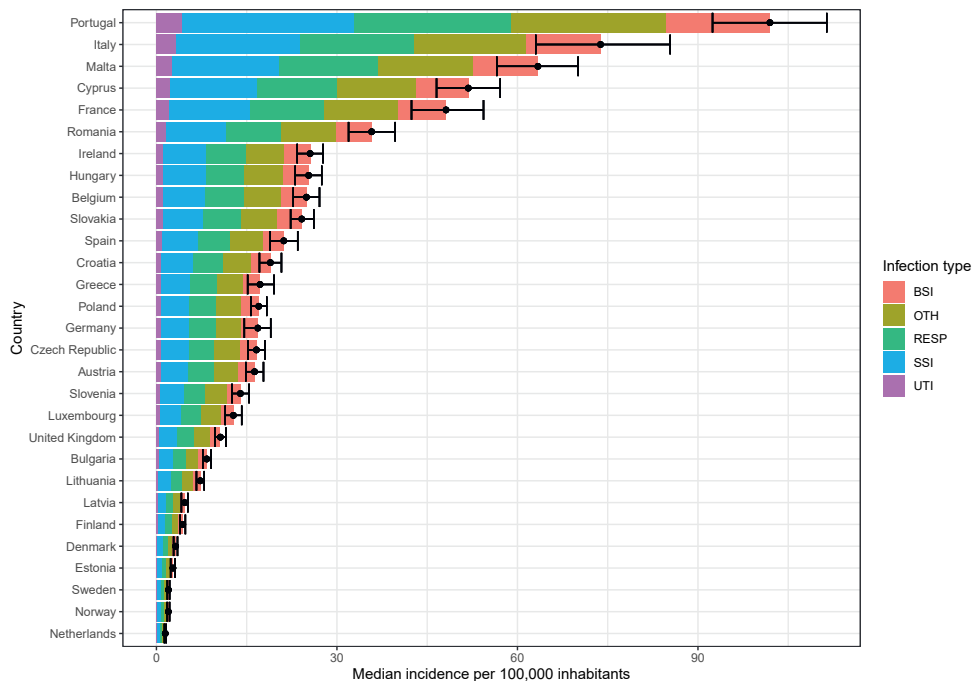


Figure 7. Median infection incidence in 29 European countries due to MRSA in 2015 as estimated by Cassini *et al.* for five infection types (colours) and total (point) with 97.5-102.5% confidence ranges.

The five infection types are: bloodstream infections (BSI), urinary tract infections (UTI), respiratory tract infections (RESP), surgical site infections (SSI), and other infections (OTH).

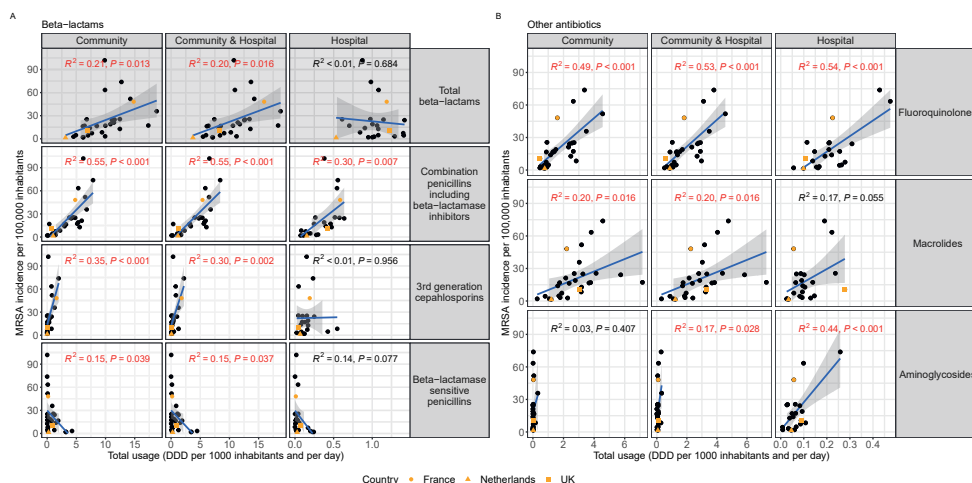


Figure 8. Antimicrobial usage in 29 European countries is associated with MRSA infection incidence.

A. Beta-lactam (ATC code classes: "J01C" and "J01D") usage in the community, hospitals or combined. B. For other antibiotics, associations were seen with fluoroquinolones ("J01MA"), Macrolides ("J01FA") and aminoglycosides ("J01G") (here the outlier of Portugal was excluded). See Supplementary data for all antibiotics. Significant trends are highlighted with a red R^2 and p-value ($p < 0.05$). Shaded cells indicate summary classes of antibiotics – those that are sums of other columns (see Supplementary Table S5). Shaded areas around the blue trend line are the 95% confidence level interval for predictions from a linear model.

Discussion

In this project we brought together isolates from three European countries with varying MRSA incidence. Surprisingly, we found that harmonizing a definition and unifying data analysis for epidemic success was extremely challenging. We explored the internationally differing MRSA surveillance programs, strain collections, typing methods and uses of data⁸. THD analysis confirmed that our operational definitions of success did not completely capture epidemic success between countries. A unified framework for MRSA sampling is needed to establish cohesive sample and data collections⁸.

The high variation in MRSA incidence and in CC types in European countries indicates differing selection pressures based on geography. Identifying markers of selection in successful isolates in different countries will be key to designing effective interventions to reduce selection.

Comprehensive phylogenetic analysis by GWAS did not identify mutations associated with success. Despite using pyseer, the complex lineage structure of MRSA may have confounded the analysis. This method does not include most of the antibiotic resistances which in MRSA are due to resistance genes carried on mobile genetic elements. A THD analysis was more nuanced assigning indices for success. This approach allowed CC22 to clearly be identified as the most successful clone, despite samples being chosen across a range of CCs.

Across the collection, an antibiotic resistance phenotype was associated with THD success for ciprofloxacin, macrolides and mupirocin (Figure 6). Success was not associated with gentamicin, rifampicin and trimethoprim resistance. This pattern

does not simply align with CC22 AMR profiles, or with resistance profiles in the UK MRSA, and demonstrates that CCs across the strain collection and all three countries contributed to this finding.

If certain antimicrobial resistances were particularly associated with success, we might expect the antimicrobials to be used at higher frequency in areas where these resistances have become prevalent. We used standardised data and estimates across Europe to answer this question. Firstly, we demonstrated that β -lactam use was correlated with MRSA incidence and specifically, this could be narrowed down to penicillins combined with β -lactam inhibitors in both hospitals and the community. Additionally, there was correlation with 3rd generation cephalosporin use in the community. Importantly, higher usage of β -lactamase sensitive penicillins in the community was correlated with a lower MRSA incidence, likely due to their effectiveness when MRSA incidence is low. These results may provide suggestions as to which beta -lactams could be targeted by stewardship interventions and in which locations.

Beyond β -lactams, fluoroquinolone use (including ciprofloxacin) correlated with MRSA incidence across Europe. Resistance to fluoroquinolones due to stable point mutations is common in successful clones, and reduction of ciprofloxacin usage has previously been implicated in MRSA incidence decline in UK hospitals ⁹, while resistance was identified as a key selected epidemiological marker using phylogenetic methods ^{24,37}. Fluoroquinolone antimicrobials are particularly secreted onto the skin and mucous membranes ³⁸, influencing the colonising microbiome, and presumably selecting a host reservoir of MRSA.

Macrolide resistance was also implicated as having association with successful MRSA, despite the instability of the resistance gene in MRSA populations ^{9,37,39}. Genes are typically carried on plasmids and transposons with high incidence of gain and loss in experimental and phylogenetic studies ²³. High frequency of resistance in MRSA suggests active selection. Usage of macrolides in the community correlated with MRSA incidence in Europe and further studies should focus on the proportion of resistant MRSA which may vary across countries.

While mupirocin resistance was identified as a marker of success, the incidence is relatively low. Similarly, *tst* gene (encoding for the Toxic shock syndrome toxin (TSSST)) carriage was implicated in success, but incidence was also low ⁴⁰. Aminoglycoside resistance was less prevalent in successful MRSA, though use in hospitals was associated with MRSA incidence, and we can speculate the large plasmids carrying such resistances may be a burden to colonising strains. Rifampicin resistance mutations are also rare, possibly due to fitness cost ⁴¹.

MRSA isolates showed evidence of recent spread from one country to others, but limited spread in the new location. However, the study was hampered by under-sampling to evidence this. Our recent mathematical modelling has suggested that dominant local clones have a particular advantage in outcompeting introduced clones, particularly when antimicrobial resistance genes are unstable ¹⁰.

All epidemiological studies are limited and biased by the isolates chosen to study. Here we attempted to power our study by selecting successful versus unsuccessful/sporadic isolates, which was hampered by un-harmonized collections. The choice of isolates may have skewed our THD analysis. THD is a method that benefits from large strain collections, and future studies could investigate global populations assigning success and utilising comparisons with genotypic AMR.

This study highlights the wide variation in antimicrobial resistance incidence in MRSA populations, as well as in usage of antimicrobials across Europe. There are alignments with the use of particular antimicrobial classes and MRSA infection incidence. Stewardship programmes to reduce infection incidence can be hampered if they focus on restricting antimicrobial usage that is not selective. The data presented here may allow targeted interventions, particular in locations where MRSA is prevalent. Further studies to investigate the antibiotic resistance profiles of MRSA in a wider range of locations, combined with the impact of changing antimicrobial usage over time, will support the design of enhanced stewardship interventions.

References

1. Murray, C. J. *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 (2022).
2. Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* **19**, 56–66 (2019).
3. Bode, L. G. M. *et al.* Preventing Surgical-Site Infections in Nasal Carriers of *Staphylococcus aureus*. *N. Engl. J. Med.* **362**, 9–17 (2010).
4. DeLeo, F. R., Otto, M., Kreiswirth, B. N. & Chambers, H. F. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* **375**, 1557–1568 (2010).
5. Wertheim, H. F. *et al.* The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* **5**, 751–762 (2005).
6. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
7. Stefani, S. *et al.* Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int. J. Antimicrob. Agents* **39**, 273–282 (2012).
8. Baede, V. O. *et al.* MRSA surveillance programmes worldwide: moving towards a harmonised international approach. *Int. J. Antimicrob. Agents* **59**, 106538 (2022).
9. Knight, G. M. *et al.* Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time. *J. Antimicrob. Chemother.* **67**, 2514–2522 (2012).
10. de Vos, A. S., de Vlas, S. J., Lindsay, J. A., Kretzschmar, M. E. E. & Knight, G. M. Understanding MRSA clonal competition within a UK hospital; the possible importance of density dependence. *Epidemics* **37**, 100511 (2021).
11. Gustave, C.-A. *et al.* Demographic fluctuation of community-acquired antibiotic-resistant *Staphylococcus aureus* lineages: potential role of flimsy antibiotic exposure. *ISME J.* **12**, 1879–1894 (2018).
12. Kime, L. *et al.* Transient Silencing of Antibiotic Resistance by Mutation Represents a Significant Potential Source of Unanticipated Therapeutic Failure. *MBio* **10**, e01755-19 (2019).
13. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. (2013) doi:10.48550/arxiv.1303.3997.
14. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313 (2014).
15. Seemann, T. Shovill v1.0.9 . <https://github.com/tseemann/shovill> (2020).
16. Seemann, T. Abricate. <https://github.com/tseemann/abicate> (2020).
17. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).
18. Page, A. J. *et al.* Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691–3693 (2015).
19. Lees, J. A., Galardini, M., Bentley, S. D., Weiser, J. N. & Corander, J. pyseer: a comprehensive tool for microbial pangenome-wide association studies. *Bioinformatics* **34**, 4310–4312 (2018).
20. Jaillard, M. *et al.* A fast and agnostic method for bacterial genome-wide association studies: Bridging the gap between k-mers and genetic events. *PLOS Genet.* **14**, e1007758 (2018).

21. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Antimicrobial susceptibility testing EUCAST disk diffusion method Version 7.0. https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents (2019).
22. Rasigade, J.-P. *et al.* Strain-specific estimation of epidemic success provides insights into the transmission dynamics of tuberculosis. *Sci. Rep.* **7**, 45326 (2017).
23. Wirth, T. *et al.* Niche specialization and spread of *Staphylococcus capitis* involved in neonatal sepsis. *Nat. Microbiol.* **5**, 735–745 (2020).
24. Wirth, T., Wong, V., Vandenesch, F. & Rasigade, J. Applied phyloepidemiology: Detecting drivers of pathogen transmission from genomic signatures using density measures. *Evol. Appl.* **13**, 1513–1525 (2020).
25. Glaser, P. *et al.* Demography and Intercontinental Spread of the USA300 Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Lineage. *MBio* **7**, e02183-15 (2016).
26. Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82**, 1–26 (2017).
27. R Core Team. R: A language and environment for statistical computing. (2017).
28. Rasigade, J.-P. Time-scaled haplotypic density (THD). <https://github.com/rasigadelab/thd> (2020).
29. Rasigade, J.-P. *et al.* *Staphylococcus aureus* CC30 Lineage and Absence of *sed,j,r*-Harboring Plasmid Predict Embolism in Infective Endocarditis. *Front. Cell. Infect. Microbiol.* **8**, 187 (2018).
30. Diep, B. A. *et al.* Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367**, 731–739 (2006).
31. Monecke, S., Slickers, P. & Ehricht, R. Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. *FEMS Immunol. Med. Microbiol.* **53**, 237–251 (2008).
32. Ward, J. H. Hierarchical Grouping to Optimize an Objective Function. *J. Am. Stat. Assoc.* **58**, 236–244 (1963).
33. Werkgroep Infectiepreventie (WIP). Methicilline-resistente *Staphylococcus aureus* (MRSA). <https://www.rivm.nl/documenten/wip-richtlijn-mrsa-ziekenhuizen> (2012).
34. Lekkerkerk, W. S. N. *et al.* Emergence of MRSA of unknown origin in the Netherlands. *Clin. Microbiol. Infect.* **18**, 656–661 (2012).
35. Lekkerkerk, W. S. N. *et al.* Newly identified risk factors for MRSA carriage in The Netherlands. *PLoS One* **12**, e0188502 (2017).
36. Leopold, S. R., Goering, R. V., Witten, A., Harmsen, D. & Mellmann, A. Bacterial Whole-Genome Sequencing Revisited: Portable, Scalable, and Standardized Analysis for Typing and Detection of Virulence and Antibiotic Resistance Genes. *J. Clin. Microbiol.* **52**, 2365–2370 (2014).
37. Holden, M. T. G. *et al.* A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. *Genome Res.* **23**, 653–664 (2013).
38. Høiby, N. *et al.* Excretion of ciprofloxacin in sweat and multiresistant *Staphylococcus epidermidis*. *Lancet* **349**, 167–9 (1997).
39. McCarthy, A. J. *et al.* Extensive Horizontal Gene Transfer during *Staphylococcus aureus* Co-colonization In Vivo. *Genome Biol. Evol.* **6**, 2697–2708 (2014).
40. Durand, G. *et al.* Detection of new methicillin-resistant *Staphylococcus aureus* clones containing the toxic shock syndrome toxin 1 gene responsible for hospital- and community-acquired infections in France. *J. Clin. Microbiol.* **44**, 847–53 (2006).

41. O'Neill, A. J., Huovinen, T., Fishwick, C. W. G. & Chopra, I. Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrob. Agents Chemother.* **50**, 298–309 (2006).

Supplementary material

Supplementary Table S1. Phenotypic antimicrobial resistance characterisation (AMR) of MACOTRA isolate collection and genotypic profile of AMR gene and virulence gene carriage

Supplementary figures.

Supplementary data. MRSA resistance phenotype distribution by country and operational success

Supplementary data. MRSA infection incidence and Antibiotic Use

Chapter 3

MRSA surveillance programmes worldwide: moving towards a harmonised international approach

Valérie O. Baede, Michael Z. David, Arjana Tambic Andrasevic,
Dominique S. Blanc, Michael Borg, Grainne Brennan, Boudewijn Catry,
Aurélié Chabaud, Joanna Empel, Hege Enger, Marie Hallin,
Marina Ivanova, Andreas Kronenberg, Kuntaman Kuntaman,
Anders Rhod Larsen, Katrien Latour, Jodi A. Lindsay, Bruno Pichon,
Dewi Santosaningsih, Leo M. Schouls, François Vandenesch,
Guido Werner, Dorota Žabicka, Helena Žemličková,
Harald Seifert, Margreet C. Vos

for MRSA surveillance worldwide study group (ISAC),
ESCMID Study Group for Nosocomial Infections,
ESCMID Study Group for Staphylococci and Staphylococcal Diseases

International Journal of Antimicrobial Agents. 59, 106538 (2022)

DOI: [10.1016/j.ijantimicag.2022.106538](https://doi.org/10.1016/j.ijantimicag.2022.106538)

Abstract

Multinational surveillance programmes for methicillin-resistant *Staphylococcus aureus* (MRSA) are dependent on national structures for data collection. This study aimed to capture the diversity of national MRSA surveillance programmes and propose a framework for harmonisation of MRSA surveillance.

The International Society of Antimicrobial Chemotherapy (ISAC) MRSA Working Group conducted a structured survey on MRSA surveillance programmes and organised a webinar to discuss the programmes' strengths and challenges and guidelines for harmonisation.

Completed surveys represented 24 MRSA surveillance programmes in 16 countries. Several countries reported separate epidemiological and microbiological surveillance. Informing clinicians and national policymakers were the most common purposes of surveillance. Surveillance of bloodstream infections (BSI) was present in all programmes. Other invasive infections were often included. Three countries reported active surveillance of MRSA carriage. Methodology and reporting of antimicrobial susceptibility, virulence factors, molecular genotyping and epidemiological metadata varied greatly.

Current MRSA surveillance programmes rely upon heterogeneous data collection systems, which hampers international epidemiological monitoring and research. To harmonise MRSA surveillance, we suggest improving the integration of microbiological and epidemiological data, implementation of central biobanks for MRSA isolate collection, and inclusion of a representative sample of skin and soft tissue infection cases in addition to all BSI cases.

Introduction

Antimicrobial resistance (AMR) is one of the greatest threats to public health. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the second most common cause of antibiotic-resistant bacterial infection in the European Union (EU) and European Economic Area (EEA) ¹. Many MRSA originate from a limited number of historically dominant clonal lineages ². While some MRSA clones are found worldwide, others are restricted to certain geographic areas, implying differences in transmission ³. To analyse MRSA transmission and to decrease the incidence of new infections, international epidemiological research is crucial, and this research depends on MRSA surveillance programmes.

Many MRSA surveillance programmes exist worldwide, but only a few are multinational ⁴. One European multinational programme is the European Antimicrobial Resistance Surveillance Network (EARS-Net) ⁵. EARS-Net is coordinated by the European Centre for Disease Prevention and Control (ECDC) and depends on national surveillance systems. While susceptibility testing and interpretation recommendations have been harmonised (EUCAST) ⁵, national surveillance programmes use different sampling strategies and laboratory techniques that can bias analyses ⁶. Also, non-European multinational MRSA surveillance programmes mostly depend on national networks using different methodologies. Examples are the Asian Network for Surveillance of Resistant Pathogens (ANSORP), the Latin American Network for Antimicrobial Resistance Surveillance (ReLAVRA), the SENTRY Antimicrobial Surveillance Program and the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.), now embedded in the Antimicrobial Testing Leadership and Surveillance (ATLAS) database ^{7–11}.

Heterogeneity in testing and sampling practices hampers international epidemiological surveillance and the establishment of an early warning system for emerging MRSA clones ^{4,12,13}. Additionally, it lowers the quality of available data. This can be illustrated by the experiences of the MACOTRA study group, which aimed to establish an MRSA strain collection to analyse transmission success of MRSA. However, drafted definitions of successful versus unsuccessful MRSA strains were not applicable due to the heterogeneity described above. As a result, multiple strategies for strain selection were adopted, leading to selection bias and decreased data comparability. This demonstrates that the current organisation of MRSA surveillance systems and reference laboratories are not sufficient to support a greater understanding of MRSA transmission, nor to detect emerging, virulent strains.

The aim of this project was to capture the diversity of existing national and institutional MRSA surveillance programmes and propose a framework for a standardised (inter)national surveillance network. A structured survey on current MRSA surveillance practices was conducted, followed by a webinar organised by the International Society of Antimicrobial Chemotherapy (ISAC) MRSA Working Group.

Methods

ISAC MRSA Working Group members were contacted to identify directors or head microbiologists of national or regional MRSA surveillance programmes or staphylococcal reference laboratories in their respective countries. Other representatives of national organisations participating in EARS-Net were contacted directly ⁵. All representatives were invited to participate in a structured survey drafted by the executive committee of the ISAC MRSA Working Group (MCV (chair), MZD, HS,

VB, SS). This survey contained sections about organisational structure, surveillance goals, strain and sample characteristics, epidemiological metadata and laboratory reports. An overview of the survey is given in supplementary data.

Additionally, surveillance programme representatives were invited to participate in a webinar, held on 10 March 2021, organised by the ISAC MRSA Working Group and the MACOTRA study group, which was entitled: 'Regional and National MRSA Surveillance Programs Worldwide: Results of a Survey and Discussion of Current Practices'. Its purpose was to present an overview of surveillance programmes to an international audience, discuss these programmes' strengths and challenges, and discuss the requirements for harmonisation of MRSA surveillance.

Results

Representatives of 12 MRSA surveillance programmes in 9 countries were invited through the ISAC MRSA Working Group (Figure 1). Another 21 national organisations participating in EARS-Net were also invited. In total, 18 surveys were completed between January and April 2021, representing 24 MRSA surveillance programmes in 14 European and 2 non-European countries. Multiple surveillance programmes were described for Belgium (3), Germany (3), France (2), Indonesia (2), Switzerland (2) and the United States of America (USA) (2). Fourteen surveillance programmes in 8 countries were presented at the webinar.

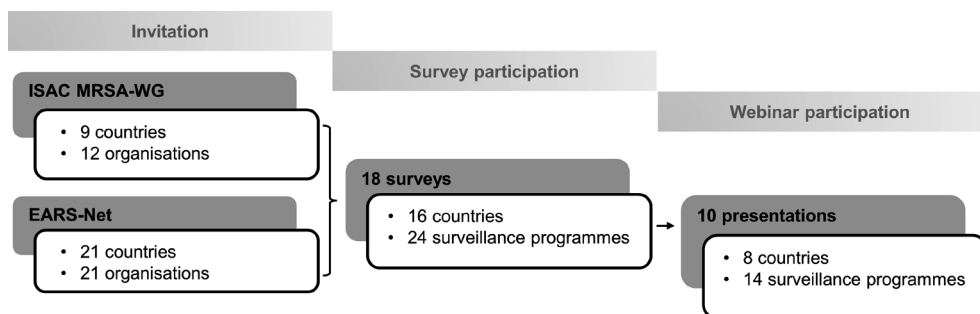


Figure 1. Overview of participating surveillance programmes

Representatives of MRSA surveillance programmes were identified through the network of the ISAC MRSA working group (ISAC MRSA-WG) or through the participation in the European Antimicrobial Resistance Surveillance Network (EARS-Net). Listed are the numbers of contacted organisations and respective number of countries. Also listed are the number of returned surveys and presentations given at the webinar, for the respective number of included countries and surveillance programmes.

Survey

A summary of survey results is given in Table 1.

	BE	CH-1	CH-2	CZ	DE	DK	EE	FR	GB	HR	ID-1	ID-2	IE	MT	NL	NO	PL	US
Surveillance structure																		
MRSA surveillance standardised	x		x		x	x	x	x	x	x				x	x	x		x
MRSA surveillance on national level	x		x	x	x	x	x	x	x	x	x	x	x	999	x	x	x	x
MRSA surveillance on regional level		x	x		x	x	U					U		999	x			x
MRSA surveillance on local/hospital level	x	x	x	x	x	x	x		x	x	x	x	x	x	x		U	x
General community included			x			x		x	x	x		x	x		x	x		x
Outpatient clinics included			x			x	x	x		x			x	x	x	x		
Mandatory for specific communities	x					x												
Mandatory for specific anatomic sites or infections (e.g. BSI)	x			x	x	x	x		x	x	x		x			x		x
Results reported in an annual report, scientific publications, website etc.	x		x	x	x	x	x	x	x	x	x		x	x	x	x		x
Organisational structure																		
SRL is a governmental organisation				x	x	x	x	x	x	x	x				x	x	x	x
No extra costs for genotyping	x			x	x	x		x	x	x			x	999	x	x	x	x
No extra costs for other tests	x			x	x	x		x	x	x			x	999	NA	x	x	x
Mandatory submission of strains						x				x						x		
Purpose of surveillance																		
ECDC data collection	x			x	x	x	x	x	U	x			x	x	x		x	
National epidemiology	x		x		x	x	x	x	x	x	x			x	x	x	x	x
Clinical question	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x

Table 1. Continued on next page

	BE	CH-1	CH-2	CZ	DE	DK	EE	FR	GB	HR	ID-1	ID-2	IE	MT	NL	NO	PL	US
Research question, e.g. virulence factors	x	x		x	x	x		x	x			x	x		x	x	x	
Research question, e.g. molecular typing	x	x		x	x	x		x	x			x	x			x	x	
Other research questions					x	x	x		x			x				x	x	
Sample data																		
MRSA collection in biobank for research	x	x		x	x	x		x	x	x		x	x	x	x	x	x	x
Infection isolates included	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Carriage isolates included	x	x	x		x	x	x		x	x		x			x	x	x	
Case/sample inclusion restricted per period	x	x		x	x				Other	x			x					x
Case/sample inclusion restricted per individual	x	x		x	x	x	U		Other	x	x		x	x	x	x	x	
Amount of MRSA isolates collected each year	100 - 1000	100 - 1000	NA	100 - 1000	1000 - 10000	1000 - 10000	NA	1000 - 10000	1000 - 10000	0 - 100	NA	NA	100 - 1000	100 - 1000	1000 - 10000	1000 - 10000	100 - 1000	100 - 1000
Sample types																		
Blood	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Wound	x	x	x	x	x	x		x	x		x	x	x	x	x	x	x	
Skin	x	x	x	x	x	x		x	x				x		x	x	x	
Nose/throat/perineum	x	x	x	x	x	x			x			x	x	x	x	x	x	
Other	x	x	x		x	x		x			x		x		x	x		x
Strain data																		
Default genotyping done	x	x		x	x	x		x	x			x	999		x		x	x
WGS	x	x			x	x		x	x						x	x	x	x
MLST	x			x											x	x	x	x
<i>spa</i>	x			x	x	x							x			x	x	x

Table 1. Continued on next page

	BE	CH-1	CH-2	CZ	DE	DK	EE	FR	GB	HR	ID-1	ID-2	IE	MT	NL	NO	PL	US
PFGE	x	x															x	
Other genotyping technique	x	x				x							x		x		x	x
Antimicrobial susceptibility tested	x		x	x	x	x	x	x	Other	x	x	x	x	999		x	x	x
Virulence factors tested	x	x			x	x		x	x				x	999	x	x	x	x
Recorded epidemiological metadata																		
Age			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Gender			x	x	x	x	x		x	x	x	x		x	x	x	x	x
Date of sampling	x	x		x	x	x	x	x		x	x	x	x	x	x	x	x	x
Place of residence		x	x	x		x	x		x	x	x	x	x	x	x	x	x	x
MRSA acquisition risk group					x	x	999					x			x	x		x
Medical specialty		x		x	x	x	999	x	x	x	x	x	x	x	x	x		
Patient admission to ICU or general ward		x		x	x	x	999	x	x	x	x	x	x	x	x	x	x	x
Admission history in same HCC		x				x	999		x			x		NA				x
Admission history in other HCCs						x	999					x		NA				x
Outbreak metadata					x	x	999	x								x		
Recorded metadata enables outbreak traceability			U			x	999							x				
Laboratory reports																		
Laboratory reports returned to submitter	x	x	NA	x	x	x	x	x	x	x	NA	U	x	NA	x	x	x	
Turnaround time genotyping results	7d	1m	NA	3w	3w	2-7d		2w	2w		NA	U	10d	NA	2d	5d	1m	
Turnaround time virulence factor results	7d	1m	NA	1w	1w	2-7d		2w	2w		NA	U	5d	NA	2d	5d	1w	

Table 1. Continued on next page

	BE	CH-1	CH-2	CZ	DE	DK	EE	FR	GB	HR	ID-1	ID-2	IE	MT	NL	NO	PL	US
Data on laboratory reports include:																		
Genotyping result in relation to strains in the HCC	x	x			x		999		x		NA		x	NA	x		x	
Genotyping result in relation to strains in the region		x					999				NA			NA	x		x	
Genotyping result in relation to strains in the country	x	x					999	x			NA			NA	x		x	
Admission history of patients with the same genotype in the HCC							999		x		NA			NA				
Admission history of patients with the same genotype in the region							999		x		NA			NA				
Admission history of patients with the same genotype in the country							999				NA			NA				
Place of residence of patients with the same genotype							999		x		NA			NA	x			
A combination of all mentioned above, but time period restricted							999	x			NA			NA	x			

Table 1. Survey results.

For Belgium, France, Germany and the USA, results mentioned in this table described multiple surveillance programs. Details are available in the supplementary data. ID-1: this survey described MRSA surveillance at Dr Saiful Anwar Hospital in Malang, Java, Indonesia. ID-2: this survey described MRSA surveillance at Dr Soetomo Hospital, Surabaya, Java, Indonesia. CH-1: this survey described a regional MRSA surveillance program in the French part of Switzerland, coordinated by the Lausanne University Hospital. CH-2: this survey described the national AMR surveillance program ANRESIS, Switzerland. All positive answers are depicted by an X; U, Unknown; Other, other answer was applicable; NA, Not applicable; 999, missing data; blanks are negative answers. Country abbreviations: BE, Belgium; HR, Croatia; CZ, Czech Republic; DK, Denmark; EE, Estonia; FR, France; DE, Germany; ID, Indonesia; IE, Ireland; MT, Malta; NL, Netherlands; NO, Norway; PL, Poland; CH, Switzerland; GB, United Kingdom; US, United States of America.

Surveillance structure and purpose

All countries conducted surveillance at the national level, except Malta. In Malta, surveillance was performed at the sole tertiary hospital, but covered >90% of all national testing. In four countries, surveillance was primarily conducted at the hospital level and organised around the surveillance of bloodstream infections (BSI). In the Czech Republic, all hospitals performed some MRSA surveillance, and MRSA BSI surveillance captured ~80% of the population. In Ireland and Poland, passive surveillance was performed through EARS-Net participation, and several national structured surveys were conducted in the past 20 years. For Indonesia, active MRSA surveillance was performed in several hospitals, but most surveillance was conducted for research purposes.

In Belgium, France and Germany, multiple separate programmes for epidemiological and microbiological surveillance were reported. In Switzerland, a local initiative focused on molecular surveillance of MRSA exists in addition to the national surveillance system, ANRESIS, which gathers epidemiological data for all antimicrobial-resistant microorganisms. In the USA, at least two large MRSA surveillance programmes exist: a national programme on MRSA BSI in which most hospitals participate and a population-based programme of invasive MRSA infections covering ~5% of the population ¹⁴.

Most surveillance programmes served multiple goals. The most common purpose of surveillance was to inform clinicians, public health workers, and laboratories about current resistance trends (17/18). Other epidemiological goals were informing national policymakers (14/18) or EARS-Net participation (for all current EU/EEA countries except Norway). Research goals included studies on staphylococcal virulence factors (12/18), resistance profiles, specific clones such as LA-MRSA, risk factor analysis, monitoring effectiveness of interventions or outbreak investigations

Collection of isolates, microbiological and epidemiological data

Results of BSI isolates were collected in all surveillance programmes. Collection of wound (15/18), skin (12/18) or nose, throat or perineum (12/18) isolates also occurred frequently. Eleven programmes reported the inclusion of isolates from other clinical sample types, such as cerebrospinal fluid, urine, pus, sputum or all clinical samples (6/11). Active surveillance of MRSA carriage was reported only for Denmark, the Netherlands and Norway. Isolates from outpatients (9/18) and the general community (10/18) were also reported, but systematic active surveillance of these groups was performed only in Denmark, the Netherlands and Norway. Long-term storage of isolates varied, ranging from BSI isolates only to all submitted isolates. Programmes with an epidemiological focus often lacked routine isolate collection.

Most programmes collected microbiological data, such as antimicrobial susceptibilities (14/18) and the presence of virulence factors (11/18). The presence of the Panton-Valentine leukocidin (PVL) toxin was most commonly tested (8/11). Eleven programmes performed genotyping on all isolates, with *spa* typing as the most common method (6/11). A wide range of genotyping techniques were reported: whole genome sequencing (WGS) (10/11), *spa* typing (8/11), multilocus sequence typing (MLST) (6/11), pulsed-field gel electrophoresis (PFGE) (3/11), *agr* group typing (Belgium), CC398 subtyping (Denmark), MLVA (Netherlands),

MLVF (Poland), DNA microarray (Ireland), SCC*mec* typing (USA), CC8 subtyping (USA) and double locus sequence typing (local Swiss initiative).

Regarding epidemiological metadata, demographic variables were most commonly collected (16/18), followed by clinical information (14/18), MRSA risk factors (6/18) and outbreak metadata (4/18).

Webinar

The goals, strengths, challenges and future plans of ten MRSA surveillance programmes in eight countries were presented at the ISAC MRSA webinar. Strengths were the robust network of local laboratories and/or hospitals in the Czech Republic, France and Poland, as well as the national surveillance programmes in Belgium, Denmark, Germany, the Netherlands and Switzerland. In Denmark and the Netherlands, the strong collaboration between epidemiological and microbiological departments and existing WGS pipelines enhanced MRSA surveillance. However, limited collaboration between epidemiological and microbiological surveillance structures posed a major challenge for Belgium, France, Germany and Switzerland. The representatives of the Czech Republic, Denmark, Germany, the Netherlands, Poland and Switzerland advocated for the implementation of WGS as a default genotyping technique and an accompanying platform to share WGS data. For many surveillance programmes, stability of financial support was a concern.

Based on our results and webinar discussions, the ISAC MRSA Working Group, MRSA surveillance worldwide study group and the MACOTRA study group propose three suggestions to harmonise MRSA surveillance.

- Inclusion of all BSI cases and a representative number of skin and soft-tissue infection (SSTI) cases in proportion to MRSA prevalence
- Integration of microbiological and epidemiological data
- Implementation of central biobanks at the national level for the collection and further characterisation of MRSA strains using common nomenclature allowing international comparisons

The challenges and our proposal for harmonised surveillance are summarised in Figure 2.

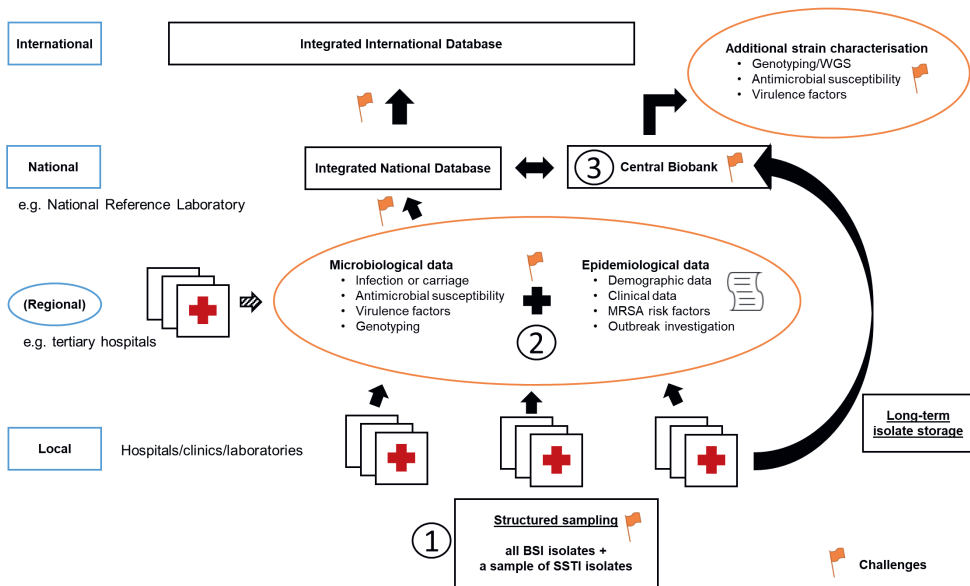


Figure 2. Proposal for harmonised MRSA surveillance

To harmonise surveillance, we propose (1) inclusion of all bloodstream infection (BSI) isolates and a representative sample of skin and soft-tissue infection (SSTI) isolates in proportion to MRSA prevalence, (2) integration of microbiological and epidemiological data in a single database using standardised report templates, and (3) implementation of central biobanks for collection and further characterisation of MRSA isolates. Orange flags depict the main challenges in harmonised surveillance.

Discussion

Our study presents an overview of existing MRSA surveillance programmes in various parts of the world with an emphasis on European countries. It demonstrates the great diversity of MRSA surveillance programmes, both in surveillance structure as well as in microbiological and epidemiological data collection. Factors potentially driving this diversity are the primary goals of surveillance, the population size, MRSA prevalence and laboratory capacity. To improve the work of these systems, a harmonised approach for surveillance programmes is needed.

We propose the inclusion of SSTI cases in addition to all BSI cases. BSI cases represent the most life-threatening MRSA infections. Because these cases are clearly defined, they provide high quality data for surveillance. Most surveillance programmes already include BSI cases.

MRSA BSIs are predominantly endogenous infections, preceded by carriage and/or non-invasive infections^{15,16}. For this reason, it is desirable to include non-BSI cases in surveillance as well. SSTIs represent the majority of *S. aureus* infections and are often acquired in the community. Inclusion of SSTIs in surveillance likely increases the probability of detecting emerging clones, which may also have significant public health impact. We recommend including a representative number of SSTI cases in proportion to BSI cases and MRSA prevalence to limit selection bias. This proportion will depend on the number of estimated MRSA BSI cases within the country, considering the expected volume and thus feasibility. A clear

definition of SSTI such as presented in the CDC/NHSN Patient Safety Component Manual must be used to prevent misclassification ¹⁷.

The integration of microbiological and epidemiological data should be improved to enhance data quality ^{4,12}. Completion of a standardised epidemiological meta-data report for each submitted case is essential. In addition to demographic data (i.e., age, gender and place of residence), the sampling date and site and classification of the isolate as being from infection or colonisation are necessary. Also required is the information on relevant risk factors for MRSA acquisition to assign the patient/carrier to a defined risk group or to identify new risk factors.

The implementation of a central MRSA biobank at the national level is needed to collect isolates corresponding to the obtained epidemiological data. Typically, this biobank would be maintained by a reference laboratory, which can provide genotyping, antimicrobial susceptibility testing and testing for virulence genes on a well-defined sample of isolates. We advocate for the use of WGS as the routine genotyping technique along with common nomenclature allowing international comparisons, and incorporate detailed phylogenetic data for local, national, and international comparisons. Furthermore, we recommend repeating the structured survey undertaken by Grundmann *et al.*, to provide an update of MRSA epidemiology at the European level ¹⁸.

We advocate that professional microbiological societies support guideline development for harmonisation. Due to its focus, aims, international representation and goals, ISAC could take the lead in this process. These guidelines should include BSI/SSTI definitions and a report template for epidemiological metadata. Additionally, a feasible ratio of BSI/SSTI cases for inclusion should be determined in collaboration with programme representatives. Furthermore, we recommend the development of an international repository for standardised surveillance data, including WGS data. Other suggestions for the harmonisation of AMR surveillance should be considered ^{4,12,19,20}, such as the alignment of surveillance goals and standardised methodology for data collection, data analysis and data sharing.

Although many countries expend substantial effort and resources on MRSA surveillance, stability of financial support is a general concern. This should be recognised in guideline development as national health budgets will greatly influence the opportunities for harmonisation of surveillance programmes.

Inclusion bias may have limited the generalisability of our study results. Nevertheless, we were able to highlight the diversity of surveillance programmes, and our webinar enabled MRSA surveillance experts to discuss their differences directly. This guided the development of our proposal for the harmonisation of MRSA surveillance programmes.

In conclusion, current MRSA surveillance programmes rely upon heterogeneous data collection, which hampers international epidemiological monitoring and research. For harmonisation of MRSA surveillance, we suggest including SSTI cases in proportion to collected BSI cases, improving the integration of microbiological and epidemiological data, implementing central biobanks for the collection and further characterisation of MRSA isolates, and genotyping of a structured sample of these isolates, preferably using WGS.

Acknowledgements

The authors thank the MRSA surveillance worldwide study group for their cooperation. The MRSA surveillance worldwide study group consists of Arjana Tambic Andrasevic, Valérie O. Baede, Dominique Blanc, Michael Borg, Grainne Brennan, Boudewijn Catry, Aurélie Chabaud, Michael Z. David, Joanna Empel, Hege Enger, Amy Gargis, Runa Gokhale, Marie Hallin, Marina Ivanova, Amelie Jouzeau, Andreas Kronenberg, Kuntaman Kuntaman, Anders Rhod Larsen, Katrien Latour, Jodi A. Lindsay, Bruno Pichon, Dewi Santosaningsih, Isaac See, Harald Seifert, Leo Schouls, Stefania Stefanj, François Vandenesch, Margreet C. Vos, Guido Werner, Dorota Žabicka, Helena Žemličková.

We are thankful to Fee Johnstone, ISAC Executive Assistant, for organisational support and Oz Golbasi and Cem Tunçel of Delta Medical Communications for technical assistance.

Additionally, we thank the MACOTRA study group for fruitful discussions. The MACOTRA study group consists of Valérie O. Baede, Anaïs Barray, Sake J. de Vlas, Anneke S. de Vos, Arya Gupta, Antoni P. A. Hendrickx, Gwenan M. Knight, Mirjam E. E. Kretzschmar, Gérard Lina, Jodi A. Lindsay, Jean-Philippe Rasigade, Leo Schouls, Mehri Tavakol, Margreet C. Vos, Willem J. B. van Wamel, Adam A. Witney.

References

1. Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* **19**, 56–66 (2019).
2. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
3. Stefani, S. *et al.* Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int. J. Antimicrob. Agents* **39**, 273–282 (2012).
4. Diallo, O. O. *et al.* Antibiotic resistance surveillance systems: A review. *J. Glob. Antimicrob. Resist.* **23**, 430–438 (2020).
5. European Centre for Disease Prevention and Control. *Surveillance of antimicrobial resistance in Europe - Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2018*. <https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2018.pdf> (2019) doi:10.2900/22212.
6. The European Committee on Antimicrobial Susceptibility Testing. EUCAST. <https://eucast.org/> (accessed June 23, 2021)
7. Song, J.-H. *et al.* Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *J. Antimicrob. Chemother.* **66**, 1061–1069 (2011).
8. Ashley, E. A. *et al.* An inventory of supranational antimicrobial resistance surveillance networks involving low- and middle-income countries since 2000. *J. Antimicrob. Chemother.* **73**, 1737–1749 (2018).
9. WHO/PAHO. Latin American Surveillance Network of Antimicrobial Resistance (ReLAVRA). <https://www.paho.org/en/topics/antimicrobial-resistance/latin-american-network-antimicrobial-resistance-surveillance> (accessed December 13, 2021)
10. Diekema, D. J., Pfaller, M. A., Shortridge, D., Zervos, M. & Jones, R. N. Twenty-Year Trends in Antimicrobial Susceptibilities Among *Staphylococcus aureus* From the SENTRY Antimicrobial Surveillance Program. *Open Forum Infect. Dis.* **6**, S47–S53 (2019).
11. Seifert, H., Blondeau, J. & Dowzicky, M. J. In vitro activity of tigecycline and comparators (2014–2016) among key WHO ‘priority pathogens’ and longitudinal assessment (2004–2016) of antimicrobial resistance: a report from the T.E.S.T. study. *Int. J. Antimicrob. Agents* **52**, 474–484 (2018).
12. Tacconelli, E. *et al.* Surveillance for control of antimicrobial resistance. *Lancet Infect. Dis.* **18**, e99–e106 (2018).
13. Grundmann, H. *et al.* A framework for global surveillance of antibiotic resistance. *Drug Resist. Updat.* **14**, 79–87 (2011).
14. Centers for Disease Control and Prevention. *Healthcare-Associated Infections - Community Interface Surveillance Report, Emerging Infections Program Network, Methicillin-Resistant Staphylococcus aureus, 2017*. <https://www.cdc.gov/hai/eip/pdf/2017-MRSA-Report-508.pdf> (accessed December 13, 2021) (2020).
15. Wertheim, H. F. L. *et al.* Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* **364**, 703–705 (2004).
16. von Eiff, C., Becker, K., Machka, K., Stammer, H. & Peters, G. Nasal Carriage as a Source of *Staphylococcus aureus* Bacteremia. *N. Engl. J. Med.* **344**, 11–16 (2001).
17. Centers for Disease Control and Prevention. National Healthcare Safety Network: Patient Safety Component. <https://www.cdc.gov/nhsn/psc/index.html> (accessed June 23, 2021) (2021).

18. Grundmann, H. *et al.* The dynamic changes of dominant clones of *Staphylococcus aureus* causing bloodstream infections in the European region: Results of a second structured survey. *Eurosurveillance* **19**, 20987 (2014).

19. World Health Organization. *Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2020*. (2020). Licence: CC BY-NC-SA 3.0 IGO

20. World Health Organization. *Emerging antimicrobial resistance reporting framework*. (2018). Licence: CC BY-NC-SA 3.0 IGO

Supplementary material

Supplementary data. Overview of survey results and data analysis

Chapter 4

Dehydration tolerance in epidemic versus nonepidemic MRSA demonstrated by isothermal microcalorimetry

Valérie O. Baede, Mehri Tavakol, Margreet C. Vos,
Gwenan M. Knight*, Willem J.B. Wamel*,
on behalf of the MACOTRA study group

*These authors contributed equally

Based on:

Microbiology Spectrum. 10, e0061522 (2022)

DOI: [10.1128/spectrum.00615-22](https://doi.org/10.1128/spectrum.00615-22)

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) clusters are considered epidemic or nonepidemic based on their ability to spread effectively. Successful transmission could be influenced by dehydration tolerance. Current methods for determination of dehydration tolerance lack accuracy. Here, a climate-controlled *in-vitro* dehydration assay using isothermal microcalorimetry (IMC) was developed and linked with mathematical modelling to determine survival of 44 epidemic versus 54 nonepidemic MRSA strains of France, United Kingdom and the Netherlands after 1 week of dehydration.

For each MRSA strain, growth parameters time to end of first growth phase ($t_{max}[h]$) and maximal exponential growth rate (μ_m) were deduced from IMC data for 3 experimental replicates, 3 different starting inocula and before and after dehydration. If the maximal exponential growth rate was within predefined margins ($\pm 36\%$ of the mean), a linear relationship between t_{max} and starting inoculum could be utilized to predict log reduction after dehydration for individual strains. With these criteria, 1330 of 1764 heat flow curves (datasets) (75%) could be analyzed to calculate the post-dehydration inoculum size and thus log reduction due to dehydration for 90 of 98 strains (92%). Overall reduction was ~ 1 log after 1 week. No difference in dehydration tolerance was found between epidemic and nonepidemic strains. Log reduction was negatively correlated with starting inoculum, indicating better survival of higher inocula.

This study presents a framework to quantify bacterial survival. MRSA strains showed great capacity to persist in the environment, irrespective of epidemiological success. This finding strengthens the need for effective surface cleaning to contain MRSA transmission.

Importance

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of infections globally. While some MRSA clusters have spread worldwide, others are not able to disseminate successfully beyond certain regions despite frequent introduction. Dehydration tolerance facilitates transmission in hospital environments through enhanced survival on surfaces and fomites, potentially explaining differences in transmission success between MRSA clusters. Unfortunately, the currently available techniques to determine dehydration tolerance of cluster-forming bacteria like *S. aureus* are labor-intensive and unreliable due to their dependence on quantitative culturing. In this study, bacterial survival was assessed in a newly developed assay using isothermal microcalorimetry. With this technique, the effect of drying can be determined without the disadvantages of quantitative culturing. In combination with a newly developed mathematical algorithm, we determined dehydration tolerance of a large number of MRSA strains in a systematic, unbiased and robust manner.

Introduction

Shortly after the introduction of methicillin into clinical use, the first methicillin-resistant *Staphylococcus aureus* (MRSA) were reported ¹. In contrast to the wide genetic variety of methicillin-susceptible *Staphylococcus aureus* (MSSA), MRSA presents a more clonal epidemiology ². Investigation of its evolutionary origin showed the emergence of MRSA as five phylogenetic distinct clones, belonging to MLST clonal complex (CC) 5, CC8, CC22, CC30, and CC45 ². Decades later, these MRSA clusters are still dominating on a global scale ³. In certain regions, historically dominant clonal clusters have been replaced by newly emerged MRSA types. One example is the replacement of the healthcare-associated CC30 EMRSA-16 by CC22 EMRSA-15 in the United Kingdom ⁴. In the American community, USA300 clone (CC8) replaced USA400 (CC1) as the most prevalent community-acquired MRSA (CA-MRSA) ⁵. These observations demonstrate the variety in MRSA transmission success. The underlying mechanisms causing these remarkable shifts in space and time are unknown.

A variety of factors could play a role in MRSA transmission success, such as genetic flexibility, interaction with the host microbiome, human behavior such as crowding, antibiotic pressure, local differences in infection prevention policies, and environmental survival. So far, attempts to explain the clonal epidemiology of MRSA have mainly focused on host-pathogen interactions, while the role of environmental survival has been largely overlooked ⁶. Nevertheless, MRSA has previously been cultured from a wide variety of surfaces and fomites in hospitals ⁷⁻¹⁰. Even after terminal cleaning practices with 500 ppm chlorine, viable MRSA or *S. aureus* was present as dry surface biofilms on surfaces in intensive care units ^{11,12}. *S. aureus* dry surface biofilms have also been found on various hospital items ¹³. This suggests a potentially important role for fomites in the spread of MRSA. In this transmission route, MRSA bacteria in bodily fluids are deposited and dehydrated on a surface, after which they can be acquired and establish themselves in a new human host. Transmission of *S. aureus* from *in-vitro* grown dry surface biofilms to hands and then to fomites has been demonstrated *in-vitro* ¹⁴. This transmission route relies on the capability of MRSA to survive dehydration and regrow in a more hospitable environment. Hence, differences in dehydration tolerance may play a role in determining whether a strain of MRSA is successful in transmission.

The results from the few studies that have investigated the role of dehydration tolerance in epidemic versus nonepidemic *S. aureus* are ambiguous ¹⁵⁻²². Furthermore, only local strain collections were considered, overall sample numbers were low, definitions of epidemiological success varied, and climate-controlled conditions were lacking. Most importantly, all these studies quantified bacterial survival by counting colony-forming units (CFU) on agar. As *S. aureus* forms grape-like clusters during growth due to incomplete separation of the daughter cells following division, both a single bacterial cell as well as a cluster of more cells will lead to the formation of a single CFU ²³. Therefore, quantification by counting CFU can largely underestimate the effect of dehydration if part of a cluster dies or alternatively overestimate this effect if very large clusters are formed. Hence, this way of quantifying the numbers of surviving bacteria is not reliable. Also, shaking, vortexing or sonication of samples is necessary to release dehydrated bacteria from any material they got deposited on. Due to poor release of the bacteria from this material, the effect of dehydration can be overestimated as well. To overcome these limitations, different techniques for bacterial quantification are needed.

Isothermal microcalorimetry (IMC) is a technique which requires no sample preparation such as sample extraction or chemical labelling. Additionally, inoculated materials can be inserted directly for measurement, without the need for bacterial detachment via vortexing or sonication. In IMC, the total heat production of all active metabolic processes in a biological sample is monitored in real time ^{24,25}. All metabolic processes produce heat, either at low levels for basic metabolism or at higher levels in the case of growth or stress responses. Earlier IMC studies have shown a linear relationship between inoculum size and lag time, represented by time of detection, in a range of bacterial species including *Escherichia coli*, *Pseudomonas putida*, *S. epidermidis*, *Proteus mirabilis*, *Lactobacillus reuteri*, and *Lactobacillus plantarum* ^{24–28}, but not, to our knowledge, for *S. aureus*, although IMC studies have explored *S. aureus* growth ^{29,30}. This linear relationship can be used to predict the size of the bacterial population based on the time of growth detection.

In this study, we describe the validation of IMC to capture *S. aureus* growth dynamics and its application to measure the survival of bacteria after dehydration. For this purpose, we combined an *in-vitro* dehydration assay using IMC with mathematical modelling to predict bacterial survival after dehydration in a quantitative manner. This assay was used to investigate the contribution of dehydration tolerance to epidemiological success in a large representative collection of curated European MRSA strains collected by the MACOTRA study group.

Methods

Strains

In this study, a total of 98 MRSA strains of the MACOTRA strain collection were investigated, including 44 epidemic (successful) and 54 nonepidemic (unsuccessful) strains. The MACOTRA strain collection was compiled to study factors explaining clonal success of MRSA. Epidemic and nonepidemic strains were defined as those from a genetic lineage with a higher or lower relative prevalence within a country see ³¹. A summary of included MACOTRA strains is given in Table 1 (see complete overview in Supplemental Table 1 (S1)). In addition, 8 well-studied MSSA and MRSA strains of different genetic background were included as pilot strains (Table 2) ³².

Culture conditions

Strains were cultured from frozen stock onto Tryptic Soy Agar supplemented with 5% sheep blood (TSA) (Becton Dickinson, Vianen, the Netherlands) at 37°C overnight. Bacterial suspensions were prepared at $OD_{600} = 1.00 \pm 0.05$ (Ultrospec 10 Cell Density Meter, Amersham Biosciences, UK) in Trypticase Soy broth (TSB) (Becton Dickinson, Vianen, the Netherlands) representing approximately 10^9 CFU/ml. Subsequently, ten-fold serial dilutions were prepared in TSB in sterile U-bottom 96-well polystyrene (PS) microplates (Greiner Bio-One GmbH, Frickenhausen, Germany).

For classical growth curves, 10 µl of this logarithmic dilution series were added to a sterile U-bottom 96-well PS microplate filled with 190 µl TSB per well. Turbidity was measured every 10 minutes by optical density (OD) at 600 nm in a microplate reader (Epoch 2, BioTek Instruments, VT, USA) for at least 20 hours. Before every measurement, the microplate was subjected to 1 minute of double-orbital shaking at low speed.

	Epidemic	Nonepidemic
Infection or carriage-related		
Infection	26	30
Carriage	12	19
Unknown	6	5
Country		
France	10	10
Netherlands	24	35
United Kingdom	10	9
Year of isolation		
2006		1
2008	8	12
2009	6	3
2013		1
2014		5
2015	5	6
2016	2	4
2017	18	18
2018	5	4
MLST-CC		
CC1	3	5
CC5	10	9
CC8	5	10
CC22	14	12
CC30	6	4
CC45	4	10
CC80		1
CC398	2	2
Other (ST59)		1
Total	44	54

Table 1. MACOTRA strain characteristics

MLST-CC: Multilocus Sequence Typing - Clonal Complexes

Climate-controlled dehydration assay using IMC

Transparent polyvinyl chloride (PVC) strips (500121 mm) (PR 107 4D, Bilcare Research GmbH, Staufen, Germany) were cut into coupons. After sterilization by autoclaving, coupons were inoculated with a 10 µl droplet of the logarithmic dilution series in duplo. To determine reference heat flow before dehydration, one set of inoculated coupons were submerged into individual microcalorimeter vials filled with rehydration medium, containing 290 µl TSB to reach a total volume of 300 µl, and placed in the isothermal microcalorimeter (calScreener™, Symcel AB, Spånga, Sweden). Microcalorimeter vials were allowed a pre-incubation period of 30 min to reach thermal equilibrium at 37 °C. Heat production of individual vials was measured as heat flow (µW) for at least 20 hours.

Strain	Genetic background	Description	Reference
Newman	ST8	MSSA, laboratory strain	43
RN6390B	ST8	MSSA, laboratory strain	44
SA2704	ST72	MSSA, clinical isolate	45
MUP15a	CC15	MSSA, clinical isolate	46
M116	CC8, ST239	MRSA, clinical isolate	47
Mu50	CC5	MRSA, clinical VISA isolate	48
RWW146	CC398	MRSA	49,50
SAC042W	CC8, USA300	MRSA, clinical isolate	51

Table 2. Pilot strain characteristics

*ST: Multilocus Sequence Type. CC: Clonal Complex

The other set of inoculated coupons was placed in a climate chamber (HPP110, Memmert GmbH + Co. KG, Büchenbach, Germany) for dehydration at 21°C, 40% relative humidity, representing an indoor environment. After dehydration for 168h, coupons were placed in prepared microcalorimeter vials containing 290 µl TSB and 10 µl H₂O (WFI for Cell Culture, Gibco, Bleiswijk, the Netherlands) and processed by IMC as described.

IMC is a highly sensitive technique, which leads to high data variability. To reduce technical variation, samples were handled following a robust protocol (details in Supplemental data analysis, section B, data cleaning 1). Obtained heat flow curves were visually inspected for the occurrence of non-typical *S. aureus* heat flow patterns suggesting contamination. Potential contaminated vial contents were cultured to confirm bacterial contamination. In case of contamination or technical issues, IMC data was excluded from further analysis (Supplemental data analysis, section B, data cleaning 1). An external baseline based on a medium blank was used to correct all obtained heat flow curves to enable data comparison between separate IMC measurements (personal communication Magnus Jansson, Symcel AB, Sweden). Data was exported with 900s intervals. Data obtained within the first 3.5 hours of measurement was excluded to guarantee thermal equilibrium ³³.

Data analysis

Comparison of heat flow curves and OD growth curves

Parallel experiments with the same bacterial dilution series of 4 pilot MSSA and MRSA strains were performed using OD and IMC. The obtained data was compared to determine if IMC data could be used to quantify key characteristics of bacterial growth. As heat flow in IMC is measured in real-time and collected for every time step, but bacterial density in OD cumulated over time, the difference in OD data was calculated for each time step to enable the comparison of these data (Supplemental data analysis, section A). The R *grofit* and *tidyverse* packages were used to fit smoothed cubic splines to heat flow curves and differentiated OD growth curves ^{34–36}. Growth parameter time to maximum heat flow in IMC or time to maximum exponential growth in OD was deduced. Spearman’s correlation coefficients were computed to compare obtained values from heat flow curves and differentiated OD curves using the *cor* function in R *stats* package ³⁵. Data of three independent replicates was used for analysis.

Extraction of growth characteristics

For further analysis of peaked time series data such as IMC data, a new algorithm was written to extract key growth parameters from the first growth phase: time to end of first growth phase ($t_{max}[h]$) and maximal exponential growth rate (μ_m) (Supplemental data analysis, section C). First, smoothed cubic splines were fitted to the heat flow curves^{34–36}. Then, the time to first peak was determined by characterizing peaks and shoulders (minor plateaus) in the data. The earliest point of the first plateau (either around a peak or shoulder) was set as the time to first peak ($t_{max}[h]$). When no plateau existed, the time to first peak was set as t_{max} . For the data up to t_{max} , a spline was fitted and the maximal exponential growth rate (μ_m) was extracted.

Heat flow analysis: prediction of log reduction

For each of the 98 MRSA strains, up to 18 datasets of growth parameters were extracted from the heat flow data from 3 experimental replicates, 3 different starting inocula, before and after dehydration for 168h. As lag time scales linearly with inoculum size, t_{max} in heat flow is linearly correlated with inoculum size under the assumption of a constant maximum exponential growth rate (μ_m). Thus, μ_m must be comparable across inocula and before and after dehydration within a replicate. This was assessed by comparing the μ_m from an individual dataset to the mean μ_m over all datasets in a replicate. Then the variation in μ_m across strains was explored. We chose to exclude a dataset if its μ_m deviated more than a chosen percentage cut-off from the mean μ_m across the replicate. A replicate was excluded if more than two of its datasets were excluded. A strain was excluded if it had only one replicate (of a possible three) remaining. The cut-off for acceptable variability was determined so it excluded the top 5% of strains with the greatest variability (Supplemental data analysis, section D, data cleaning 2). After removing these most variable strains, this cut-off was used to iteratively remove datasets: first any dataset with μ_m outside the cut-off from the mean was removed, then the mean μ_m was recalculated etc.

For the remaining data, a linear model was fitted to the t_{max} data prior to dehydration (equation 1) for each replicate to estimate replicate specific intercept (a) and gradient (b) values.

$$\text{Equation 1} \quad \log(\text{inoculum}) = a + b * t_{max}$$

Using this parameterization, the inoculum surviving dehydration could be predicted within a replicate based on the t_{max} observed after dehydration. The difference between starting inoculum and viable inoculum after dehydration was defined as log reduction. Figure 1 shows a schematic overview of data analysis. Only those replicates with a linear fit that had an $R^2 > 0.75$ and for which there were two or more than two datasets available prior to dehydration were used in further analysis (Supplemental data analysis, section E, data cleaning 3 & 4).

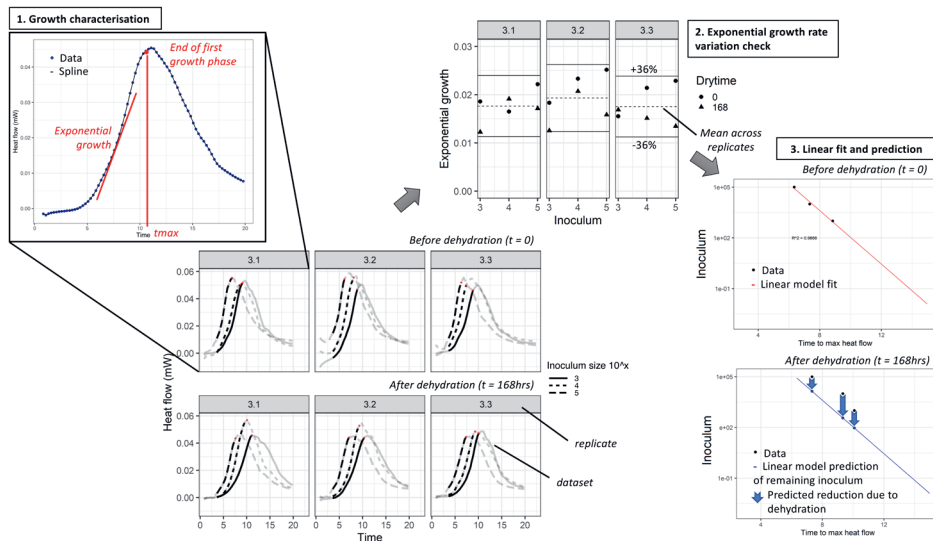


Figure 1. Schematic overview of all steps in data analysis

In the first step, smoothed cubic splines were fitted to the data and key growth parameters were extracted from the first growth phase of all datasets (e.g. t_{max} and maximal exponential growth rate shown in red here). At step 2, datasets with $\leq 36\%$ variability in maximal exponential growth rate were included for further analysis. In step 3, a linear model was fitted to t_{max} data prior to dehydration and the inoculum surviving dehydration was predicted based on the t_{max} after dehydration.

Unless otherwise stated, all values are reported as the mean for the strain, which is the mean over the replicates (up to three) of the mean log reduction over all inocula in a replicate. Fewer than three replicates would remain in the final analysis if datasets had been removed in any of the above four data cleaning steps.

During all steps of data analysis, strain metadata were blinded for the executing researcher (GMK).

Statistical analysis

For statistical analysis of log reduction, a linear mixed-effects model was built using the R *lme4* and *lmerTest* packages^{37,38}. Epidemiological success and starting inoculum were taken as fixed effects. To account for selection bias, genetic lineage, and originating country were taken as random effects.

Data availability

All strain metadata, OD and IMC data, and accompanying analysis code are available from the GitHub repository (https://github.com/gwenknight/strain_growth).

Results

Validation of IMC for *S. aureus* growth characterization and quantification

For validation of IMC in *S. aureus* growth characterization, OD growth curves and heat flow curves were compared in parallel experiments. Growth parameters were determined and a strong correlation was found for the growth parameter t_{max} across the two data types (deduced as time to first peak in heat flow and time to maximum exponential growth in OD) for all four strains ($r > 0.95$) (Figure 2). This indicates that heat flow curves obtained by IMC represent classical bacterial growth curves measured by OD under the study conditions, at least until the first peak in heat flow. Therefore, further data analysis was limited to the initial growth phase of the heat flow curve.

Next, heat flow curves were determined for 10-fold serial dilutions of 8 pilot MSSA and MRSA strains. Typically, *S. aureus* heat flow curves are characterized by a bell-like shape, usually reaching maximum heat flow within 5 hours after lag time and declining to a stable heat flow level within a similar time period. Within this characteristic IMC profile, biological variation was seen with each pilot strain displaying a unique kinetic fingerprint, shown by the strain-specific shape of obtained heat flow curves (examples shown in Figure 3A and 3B). The observed profiles were comparable for different starting inocula of a strain, although occasionally

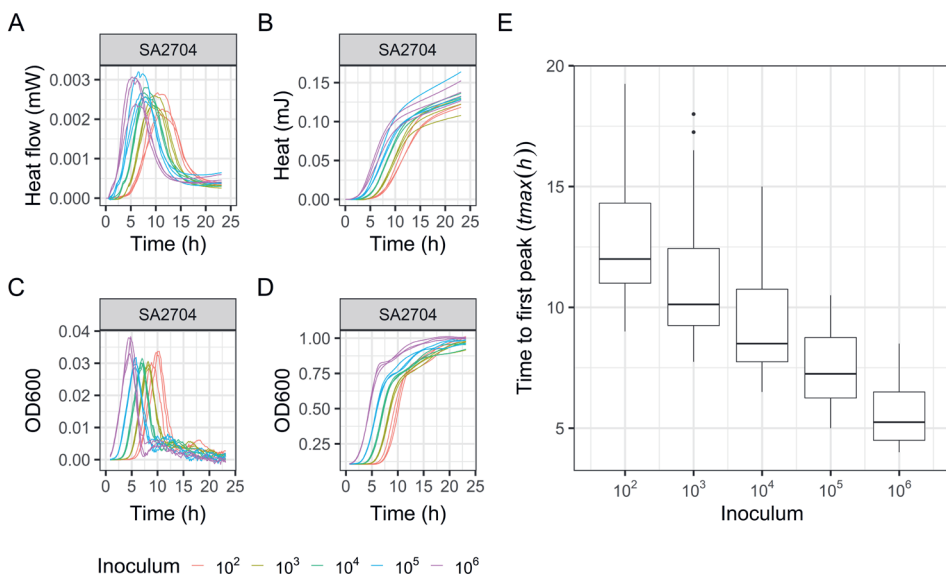


Figure 2. Validation of IMC for *S. aureus* growth characterization

Growth curves were deduced from heat flow data (A: raw data or B: cumulated over time) and optical density data (C: differentiated between time steps or D: raw data) (example strain SA2704). The correlation between t_{max} from heat flow or OD and inoculum size for strains SA2704, SAMUP15a, M116 and SAC042W was 0.97, 0.96, 0.96 and 0.95 respectively (based on 3 independent experiments, $p < 0.001$). Panel E shows the linear relationship between inoculum and t_{max} for different starting inocula for 8 pilot MSSA and MRSA strains (see Supplemental data analysis, section A for underlying data). Data from 3 independent experiments.

decreased values for maximum heat flow and exponential growth rate were observed in lower starting inocula. However, we found an inoculum-dependent lag time, i.e. a longer lag time for lower starting inocula leading to a later heat flow peak. Based on these data, a linear relationship between inoculum size and t_{max} was confirmed for *S. aureus* (Figure 2) as was seen earlier for other bacteria 24–26,28.

Together, these findings validated the use of IMC for *S. aureus* growth characterization and quantification.

MRSA dehydration tolerance

Heat flow curves were obtained for 98 MRSA strains before and after dehydration. Various steps of data cleaning were performed (Supplemental table S2), resulting in a final value of 1330 datasets (75%) for 98 strains. Log reduction after dehydration could be determined for 90 strains (92%).

Overall, log reduction after 168 hours of dehydration was 0.91 (SD = 0.44). For epidemic strains, the mean log reduction over 168h of dehydration was 0.92 (SD = 0.44). For nonepidemic strains, this was 0.95 (SD = 0.57). This difference was not significant ($t = -0.34$, $p > 0.05$). Log reduction varied significantly ($F = 11.35$, $p < 0.001$) by starting inoculum from 1.14 (SD = 0.65) for 10^3 , to 1.00 (SD = 0.59) for 10^4 to 0.72 (SD = 0.53) for 10^5 (here mean was taken over replicates and then per inoculum) (Figure 4A). A *post hoc* Tukey multiple pairwise comparison test showed significant difference between 10^5 and the two lower starting inocula ($p < 0.05$), but not between starting inocula 10^4 and 10^3 . There was a similar trend in smaller reduction with higher inoculum across epidemic and nonepidemic strains (Figure 4B) and lineages (Figure 4C). A trend towards higher dehydration tolerance was found for epidemic strains of CC8 (Figure 4C). Furthermore, a trend towards lower dehydration tolerance was found for UK CC22 strains compared to

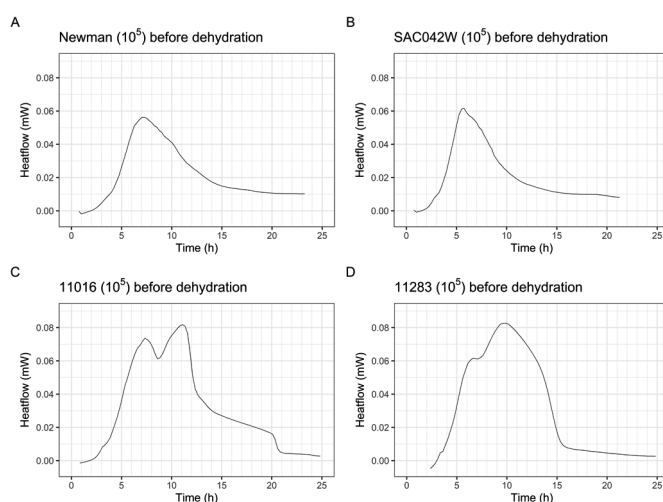


Figure 3. Heat flow profiles

Typically, *S. aureus* heat flow curves are characterized by a bell-like shape (examples in panel A and B). In 31 strains, multiple datasets with multiple heat flow peaks were observed (examples in panel C and D).

the French and Dutch CC22 isolates (Figure 4D). Additional results on the linear relationship between inoculum and *t*_{max} for all strains, within-strain variation of log reduction and the association between log reduction, starting inoculum, epidemiological success, and country are given in Supplemental Figures 7-9.

Statistical model results

In order to test for a difference in dehydration tolerance between epidemic and nonepidemic MRSA while accounting for starting inoculum, genetic lineage, and country of origin, a linear mixed-effects model was used. The effect size of epidemiological success was too small to explain differences in log reduction due to dehydration ($b = -0.07$, 95% confidence interval (CI) = $-0.22 - 0.07$, $p = 0.31$), i.e. no difference was found between the epidemic and nonepidemic MRSA strains. Differences in log reduction were explained by different starting inocula ($p < 0.001$), with an effect size of -0.21 (95% CI = $-0.29 - -0.12$).

Discussion

In this study, dehydration tolerance was explored for 98 MRSA strains with different epidemiological characteristics using a newly developed assay. No difference in dehydration tolerance was found between epidemic or nonepidemic strains of MRSA. Overall, we observed an average reduction of only approximately 1 log

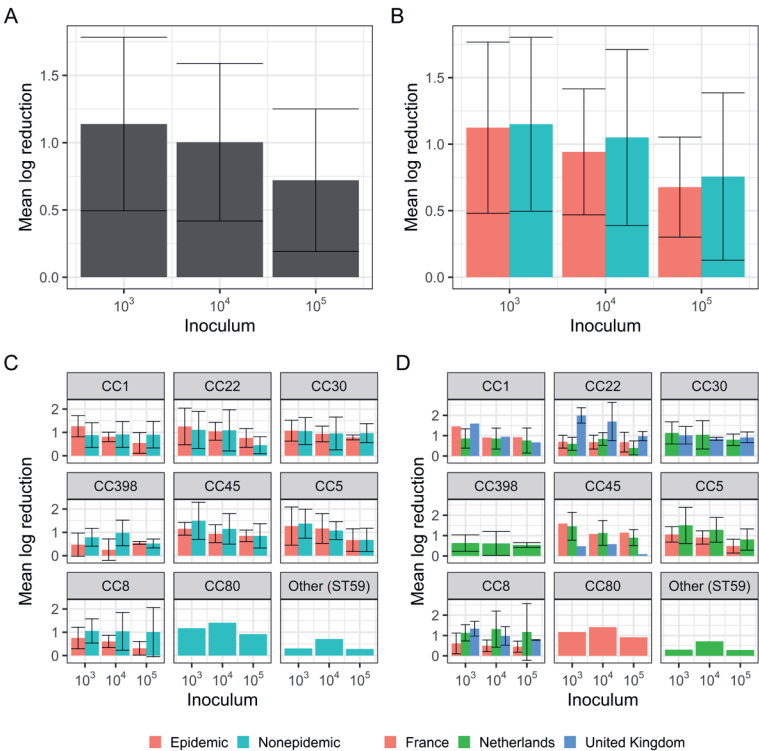


Figure 4. Mean log reduction results

Mean log reduction by (A) inoculum, (B) inoculum and success (color), (C) Inoculum, success (color) and lineage (panel), (D) Inoculum, country (color) and lineage (panel). Bars are mean with standard deviation error bars.

bacteria after 7 days of dehydration, indicating that dehydration tolerance is a common characteristic in *S. aureus*.

Interestingly, we found MRSA survival was greater in higher starting inocula, indicating a bacterial density effect on survival. Chaibenjawong *et al.* has shown initial cell density dependency in desiccation tolerance for *S. aureus* lab strain SH1000³⁹. To our knowledge, our study is the first to show this for multiple clinical strains of *S. aureus* and MRSA. This finding implies greater survival of bacteria, and thus possibility for transmission, in bodily fluids with a high bacterial load such as pus. As a consequence, cleaning and disinfection protocols should be carefully implemented to ensure adequate removal of infected body fluids from the hospital environment after contamination. Equally important is compliance with hand hygiene protocols for healthcare workers, to prevent transmission through fomites.

Earlier studies investigating dehydration tolerance of MRSA in epidemic versus nonepidemic strains presented ambiguous conclusions. After 15 days of dehydration on cotton swatches, log reduction ranged between 0.2 and 1.8, which was comparable to our findings¹⁵. The same study showed that most epidemic strains lacked significant viability loss due to dehydration, but others were susceptible¹⁵. Beard-Pegler *et al.* found lower death rates for general epidemic strains compared to local epidemic strains after 7 days dehydration¹⁷. Another study found that 2 MRSA outbreak strains survived longer and in higher quantities than 3 sporadic MRSA strains, although all strains survived at least for 225 days¹⁹. In contrast, Farrington *et al.* found lower dehydration tolerance of MRSA outbreak isolates compared to isolates from the hospital environment¹⁶.

We found that dehydration tolerance was not significantly different between epidemic and nonepidemic MRSA strains. Our definition of epidemic versus nonepidemic strains, i.e. epidemiological success, was based on the relative prevalence of a genetic lineage within a country. For this, we were dependent on various surveillance programs, with different inclusion criteria and testing procedures, causing the definition of epidemiological success to vary per country and possibly affect our analyses. Also, we did not have enough statistical power to test differences in dehydration tolerance between genetic lineages. Nevertheless, our results show a trend towards high dehydration tolerance in epidemic strains of CC8. We could not confirm the findings of Knight *et al.*, which showed higher survival rates of epidemic CC22 strains after desiccation than strains of CC30, the clonal cluster it replaced as the most dominant clone in the UK²¹. In our study, UK strains of CC22 were less tolerant to dehydration than Dutch or French strains of CC22. Other studies showed higher dehydration tolerance of different clones in their specific situations. In Italy, epidemic clone ST22-IV had increased survival capabilities in various stress conditions, including dehydration, than the CC5-ST228-I clone it replaced²⁰. In the USA, clinical, colonization, and environmental *S. aureus* isolates of ST5 had higher dehydration tolerance compared to less epidemic strains of other STs in the study setting²².

Together, these findings suggest that higher dehydration tolerance might benefit clones in their adaptation to a local niche in a geographic setting. However, taking into account the overall high survival of all MRSA in our study, dehydration tolerance seems to be a universal trait of *S. aureus* contributing to the global success of MRSA.

We observed a wide range of heat flow profiles representing high biological variability across our data, shown in Figure 3&4. To account for this we performed

the analysis within strains across inocula. Lower starting inocula showed lower values for maximum heat flow and exponential growth rate. Approximately a third of strains showed substantial variability from expected strain metabolism (double peaks, wide peaks, shoulders) after the first growth phase (see example in Figure 3C and 3D). This indicated alternative metabolic processes after initial growth, supporting our analysis being limited to the initial growth phase. In some cases, this odd behavior was absent after dehydration, potentially pointing towards repression of these processes due to a stress response or survival of a subpopulation with different metabolic behavior. As the underlying mechanisms were not in the scope of this study, these were not investigated further. Additional work is needed to explore the metabolic processes and regulatory mechanisms underlying these observations and its role in *S. aureus* stress responses.

While most studies use classical CFU counts for quantification of bacterial survival, we developed a highly reproducible assay using IMC. With this technique, heat produced by viable bacteria was measured for quantification. First, the use of IMC for characterization of *S. aureus* growth was validated by the strong correlation between growth parameters in the initial growth phase derived from IMC and OD generated growth curves. For the extraction of growth parameters, we used a model-free approach by fitting smoothed cubic splines to heat flow curves. In contrast to traditional growth models, this model-free approach allows for higher flexibility to deal with biological variation^{34,40}. Additionally, we confirmed a linear relationship between time to end of first growth phase (t_{max}) and starting inoculum for *S. aureus* with the requirement of a constant maximal exponential growth rate (μ_m). Based on these findings, a framework to deal with the large number of data combining an *in-vitro* dehydration assay with mathematical modelling was developed to characterize bacterial time series in order to predict bacterial survival after dehydration in a quantitative manner.

Our dehydration assay was equipped with a climate chamber ensuring climate-controlled conditions throughout the study. We chose climate conditions representing an indoor hospital environment, which is most relevant for MRSA. Higher temperatures or lower levels of relative humidity would increase the dehydration rate, which may have induced altered stress responses and therefore larger differences between strains⁴¹. Also longer dehydration periods are expected to show larger differences between strains as shown in earlier studies^{15,16,19}. Although we observed the ability of MRSA to survive after a month of dehydration under the tested conditions (data not shown), the chosen setup could successfully indicate differences between strains. As IMC is a closed system, limited oxygen availability might have influenced bacterial growth. To use IMC measurements for analysis of bacterial growth, these measurements need to be validated in comparison to an open system such as OD measurements. In our study, we confirmed a high correlation for growth parameters between both methods which validated our approach. In the hospital environment, rehydration fluids would range from spilled water to blood, pus, and other nutrient-rich bodily fluids. To ensure high reproducibility of our results, we chose to use a nutrient-rich rehydration medium in this study. The high biological variability encountered in our data led to a series of data cleaning steps to maximize data usage for our study aim. The key assumption of linearity between inoculum and lag time, required a consistent exponential growth rate across all datasets within a strain to be robust. Hence, we removed the most variable datasets (top 5%) and those for which this relationship was weak (low R^2 value). This meant that we had high confidence in the predictive ability of the relationship for the final 75% of datasets that were included for the

final data analysis and we were able to determine log reduction due to dehydration for 90 of 98 clinical MRSA strains. The developed assay could be adapted to study dehydration tolerance of other bacterial species that easily spread through the hospital environment, such as *Enterococcus faecium* and *Acinetobacter baumannii*. Additionally, IMC can be used to evaluate other stress-inducing conditions or monitor the real-time energy levels of biofilms or persisters throughout their development or treatment, as no disruptive sampling is needed.

For the analysis of these data, we developed an algorithm to extract characteristics of the first growth phase in peaked time series data, such as heat flow or the change per time step in OD. This flexible code can be used to extract similar growth characteristics for other data and allowed us to compare this large dataset of growth curves in a systematic, unbiased manner. This was the result of much interdisciplinary discussion to develop an effective tool for this microbiological question and highlights the importance and potential of interdisciplinary research.

Overall, our results show the universal capability of *S. aureus* to survive dehydration in the environment with a small effect on viable numbers. Earlier studies have shown the persistence of MRSA on hospital items and surfaces in dry biofilms, also after terminal cleaning and disinfection^{11–13,42}. This study helps by providing a well-explored open access method to quantify growth and death of bacteria under a variety of circumstances. Together, these findings urge the need for understanding survival and tolerance to environmental substances, including disinfectants.

Acknowledgements

We are grateful to all members of the MACOTRA study group for their input. We thank Dr. Jean-Philippe Rasigade for his assistance on statistical analysis. This work was part of the MACOTRA project, which was financially supported by JPIAMR 3rd call, *AMR Transmission dynamics* and Dutch ZonMw (grant no. 547001006). Gwenan M. Knight was supported by an UK MRC Skills Development Fellowship (MR/P014658/1).

The MACOTRA study group consists of Valérie O. Baede, Anaïs Barray, Sake J. de Vlas, Anneke S. de Vos, Arya Gupta, Antoni P. A. Hendrickx, Gwenan M. Knight, Mirjam E. E. Kretzschmar, Gérard Lina, Jodi A. Lindsay, Jean-Philippe Rasigade, Leo Schouls, Mehri Tavakol, Margreet C. Vos, Willem J. B. van Wamel, Adam A. Witney

References

1. Harkins, C. P. *et al.* Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biol.* **18**, 130 (2017).
2. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
3. Stefani, S. *et al.* Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int. J. Antimicrob. Agents* **39**, 273–282 (2012).
4. Wyllie, D., Paul, J. & Crook, D. Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control. *J. Antimicrob. Chemother.* **66**, 2685–2688 (2011).
5. DeLeo, F. R., Otto, M., Kreiswirth, B. N. & Chambers, H. F. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* **375**, 1557–1568 (2010).
6. Dancer, S. J. Importance of the environment in methicillin-resistant *Staphylococcus aureus* acquisition: the case for hospital cleaning. *Lancet Infect. Dis.* **8**, 101–113 (2008).
7. Boyce, J. M., Potter-Bynoe, G., Chenevert, C. & King, T. Environmental contamination due to methicillin-resistant *Staphylococcus aureus*: possible infection control implications. *Infect. Control Hosp. Epidemiol.* **18**, 622–7 (1997).
8. Bures, S., Fishbain, J. T., Uyehara, C. F. T., Parker, J. M. & Berg, B. W. Computer keyboards and faucet handles as reservoirs of nosocomial pathogens in the intensive care unit. *Am. J. Infect. Control* **28**, 465–471 (2000).
9. Lemmen, S. J., Häfner, H., Zolldann, D., Stanzel, S. & Lütticken, R. Distribution of multi-resistant Gram-negative versus Gram-positive bacteria in the hospital inanimate environment. *J. Hosp. Infect.* **56**, 191–197 (2004).
10. Neely, A. N. & Maley, M. P. Survival of enterococci and staphylococci on hospital fabrics and plastic. *J. Clin. Microbiol.* **38**, 724–6 (2000).
11. Vickery, K. *et al.* Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. *J. Hosp. Infect.* **80**, 52–55 (2012).
12. Hu, H. *et al.* Intensive care unit environmental surfaces are contaminated by multidrug-resistant bacteria in biofilms: combined results of conventional culture, pyrosequencing, scanning electron microscopy, and confocal laser microscopy. *J. Hosp. Infect.* **91**, 35–44 (2015).
13. Ledwoch, K. *et al.* Beware biofilm! Dry biofilms containing bacterial pathogens on multiple healthcare surfaces; a multi-centre study. *J. Hosp. Infect.* **100**, e47–e56 (2018).
14. Chowdhury, D. *et al.* Transfer of dry surface biofilm in the healthcare environment: the role of healthcare workers' hands as vehicles. *J. Hosp. Infect.* **100**, e85–e90 (2018).
15. Rountree, P. M. The effect of desiccation on the viability of *Staphylococcus aureus*. *J. Hyg. (Lond.)* **61**, 265–272 (1963).
16. Farrington, M., Brenwald, N., Haines, D. & Walpole, E. Resistance to desiccation and skin fatty acids in outbreak strains of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **36**, 56–60 (1992).
17. Beard-Pegler, M. A., Stubbs, E. & Vickery, A. M. Observations on the resistance to drying of staphylococcal strains. *J. Med. Microbiol.* **26**, 251–255 (1988).
18. Wagenvoort, J. H. T. & Penders, R. J. R. Long-term in-vitro survival of an epidemic MRSA phage-group III-29 strain. *J. Hosp. Infect.* **35**, 322–325 (1997).
19. Wagenvoort, J. H. T., Sluijsmans, W. & Penders, R. J. R. Better environmental survival of outbreak vs. sporadic MRSA isolates. *J. Hosp. Infect.* **45**, 231–234 (2000).

20. Baldan, R. *et al.* Epidemic MRSA clone ST22-IV is more resistant to multiple host- and environment-related stresses compared with ST228-I. *J. Antimicrob. Chemother.* **70**, 757–765 (2015).
21. Knight, G. M. *et al.* Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time. *J. Antimicrob. Chemother.* **67**, 2514–2522 (2012).
22. Loftus, R. W., Dexter, F., Robinson, A. D. M. & Horswill, A. R. Desiccation tolerance is associated with *Staphylococcus aureus* hypertransmissibility, resistance and infection development in the operating room. *J. Hosp. Infect.* **100**, 299–308 (2018).
23. Missiakas, D. M. & Schneewind, O. Growth and Laboratory Maintenance of *Staphylococcus aureus*. in *Current Protocols in Microbiology* (John Wiley & Sons, Inc., 2013). doi:10.1002/9780471729259.mc09c01s28.
24. Braissant, O. *et al.* Isothermal microcalorimetry accurately detects bacteria, tumorous microtissues, and parasitic worms in a label-free well-plate assay. *Biotechnol. J.* **10**, 460–468 (2015).
25. Garcia, A. H., Herrmann, A. M. & Håkansson, S. Isothermal microcalorimetry for rapid viability assessment of freeze-dried *Lactobacillus reuteri*. *Process Biochem.* **55**, 49–54 (2017).
26. Maskow, T., Wolf, K., Kunze, W., Enders, S. & Harms, H. Rapid analysis of bacterial contamination of tap water using isothermal calorimetry. *Thermochim. Acta* **543**, 273–280 (2012).
27. Braissant, O., Bachmann, A. & Bonkat, G. Microcalorimetric assays for measuring cell growth and metabolic activity: Methodology and applications. *Methods* **76**, 27–34 (2015).
28. Fricke, C., Harms, H. & Maskow, T. Rapid Calorimetric Detection of Bacterial Contamination: Influence of the Cultivation Technique. *Front. Microbiol.* **10**, (2019).
29. Trampuz, A., Salzmann, S., Antheaume, J. & Daniels, A. U. Microcalorimetry: A novel method for detection of microbial contamination in platelet products. *Transfusion* **47**, 1643–1650 (2007).
30. Bonkat, G. *et al.* Standardization of isothermal microcalorimetry in urinary tract infection detection by using artificial urine. *World J. Urol.* **31**, 553–557 (2013).
31. MACOTRA Consortium. Markers of epidemiological success in European MRSA populations - Details of isolate selection. (2022) doi:10.24376/rd.sgul.19070333.v1.
32. Sultan, A. R. *et al.* Paracetamol modulates biofilm formation in *Staphylococcus aureus* clonal complex 8 strains. *Sci. Rep.* **11**, 5114 (2021).
33. Wadsö, I. *et al.* A well-plate format isothermal multi-channel microcalorimeter for monitoring the activity of living cells and tissues. *Thermochim. Acta* **652**, 141–149 (2017).
34. Kahm, M., Hasenbrink, G., Lichtenberg-Fraté, H., Ludwig, J. & Kschischo, M. grofit: Fitting Biological Growth Curves with R. *J. Stat. Softw.* **33**, 1–21 (2010).
35. R Core Team. R: A language and environment for statistical computing. (2017).
36. Wickham, H. *et al.* Welcome to the Tidyverse. *J. Open Source Softw.* **4**, 1686 (2019).
37. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, (2015).
38. Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82**, (2017).
39. Chaibenjawong, P. & Foster, S. J. Desiccation tolerance in *Staphylococcus aureus*. *Arch. Microbiol.* **193**, 125–135 (2011).
40. Braissant, O., Bonkat, G., Wirz, D. & Bachmann, A. Microbial growth and isothermal microcalorimetry: Growth models and their application to microcalorimetric data. *Thermochim. Acta* **555**, 64–71 (2013).

41. Potts, M. Desiccation tolerance of prokaryotes. *Microbiol. Rev.* **58**, 755–805 (1994).
42. Almatroudi, A. *et al.* *Staphylococcus aureus* dry-surface biofilms are not killed by sodium hypochlorite: implications for infection control. *J. Hosp. Infect.* **93**, 263–270 (2016).
43. Duthie, E. S. & Lorenz, L. L. Staphylococcal Coagulase: Mode of Action and Antigenicity. *Microbiology* **6**, 95–107 (1952).
44. Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J. & Schlievert, P. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J. Bacteriol.* **170**, 4365–4372 (1988).
45. Wertheim, H. F. L. *et al.* Associations between *Staphylococcus aureus* Genotype, Infection, and In-Hospital Mortality: A Nested Case-Control Study. *J. Infect. Dis.* **192**, 1196–1200 (2005).
46. van Trijp, M. J. C. A. *et al.* Genotypes, superantigen gene profiles, and presence of exfoliative toxin genes in clinical methicillin-susceptible *Staphylococcus aureus* isolates. *Diagn. Microbiol. Infect. Dis.* **66**, 222–224 (2010).
47. Sultan, A. R. *et al.* Production of Staphylococcal Complement Inhibitor (SCIN) and Other Immune Modulators during the Early Stages of *Staphylococcus aureus* Biofilm Formation in a Mammalian Cell Culture Medium. *Infect. Immun.* **86**, (2018).
48. Kuroda, M. *et al.* Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240 (2001).
49. Slingerland, B. C. G. C. *et al.* Survival of *Staphylococcus aureus* ST398 in the Human Nose after Artificial Inoculation. *PLoS One* **7**, e48896 (2012).
50. McCarthy, A. J. *et al.* *Staphylococcus aureus* CC398 Clade Associated with Human-to-Human Transmission. *Appl. Environ. Microbiol.* **78**, 8845–8848 (2012).
51. den Reijer, P. M. *et al.* Detection of Alpha-Toxin and Other Virulence Factors in Biofilms of *Staphylococcus aureus* on Polystyrene and a Human Epidermal Model. *PLoS One* **11**, e0145722 (2016).

Supplemental material

Supplemental data analysis

Supplemental Table S1. MACOTRA strain metadata

Supplemental Table S2. Data cleaning steps

Chapter 5

The survival of epidemic and sporadic MRSA on human skin mimics is determined by both host and bacterial factors

Valérie O. Baede, Michella M. Voet, Tanny J. K. van der Reijden,
Annelies van Wengen, Deborah E. Horst-Kreft,
Nicole A. Lemmens-den Toom, Mehri Tavakol, Margreet C. Vos,
Peter H. Nibbering*, Willem J.B. Wamel*

*These authors contributed equally

Epidemiology & Infection. 150, e203 (2022)

DOI: [10.1017/s0950268822001765](https://doi.org/10.1017/s0950268822001765)

Summary

Bacterial survival on, and interactions with, human skin may explain the epidemiological success of MRSA strains. We evaluated the bacterial counts for 27 epidemic and 31 sporadic MRSA strains on 3D epidermal models based on N/TERT cells (NEMs) after 1, 2 and 8 days. In addition, the expression of antimicrobial peptides (hBD-2, RNase 7), inflammatory cytokines (IL-1 β , IL-6) and chemokine IL-8 by NEMs was assessed using immunoassays and the expression of 43 *S. aureus* virulence factors was determined by a multiplex competitive Luminex assay. To explore donor variation, bacterial counts for five epidemic and seven sporadic MRSA strains were determined on 3D primary keratinocyte models (LEMs) from three human donors. Bacterial survival was comparable on NEMs between the two groups, but on LEMs, sporadic strains showed significantly lower survival numbers compared to epidemic strains. Both groups triggered the expression of immune factors. Upon interaction with NEMs, only the epidemic MRSA strains expressed pore-forming toxins, including alpha-hemolysin (Hla), gamma-hemolysin (HlgB), PVL (LukS) and LukED. Together, these data indicate that the outcome of the interaction between MRSA and human skin mimics, depends on the unique combination of bacterial strain and host factors.

Introduction

A large part of the general population is a persistent or intermittent carrier of *Staphylococcus aureus* ¹. *S. aureus* is an opportunistic pathogen and carriage increases the risk of subsequent infection ¹ ranging from skin and soft-tissue infections (SSTI) to invasive life-threatening infections, such as endocarditis and sepsis ¹. Treatment of these infections is hindered by the emergence of methicillin-resistant *S. aureus* (MRSA), and especially community-acquired (CA)-MRSA which has become a significant cause of SSTI worldwide ².

MRSA emerged originally as a limited number of clonal complexes (CCs), and these still represent the global dominant clusters today ^{3,4}. Some examples are ST5-MRSA-II (CC5), ST239-MRSA-III (CC8) and ST8-MRSA-IV (CC8), also known as USA300 ^{2,4}. On occasion, dominant clusters are replaced by others. In the United Kingdom, a shift in dominant MRSA clusters occurred between 2001 and 2007, from EMRSA-16 (CC30) to EMRSA-15 (CC22) ⁵. Likewise in North America, USA300 replaced the former dominant USA400 (CC1) and became the major cause of SSTI within five years after its emergence ^{2,6}.

While epidemic strains become dominant due to successful transmission, sporadic strains fail to disseminate widely despite similar geographical and societal circumstances for transmission. The epidemiological success of USA300 has been studied extensively. Suggested success factors are increased virulence through the production of toxins, such as alpha-toxin and Panton-Valentine leucocidin (PVL), the uptake of the arginine catabolic mobile element (ACME), and the acquisition of fluoroquinolone resistance ⁶⁻⁹. However, while these factors may have advanced transmission of USA300, they do not fully explain its success ⁶.

In addition to increased virulence, host-pathogen interactions may have influenced the epidemiological success of MRSA strains. Since *S. aureus* carriage on skin increases the risk of subsequent infection and transmission to others, its ability to survive on skin forms the basis of successful transmission ^{1,10}. Nevertheless, it remains unknown whether epidemic MRSA are more capable of survival on the skin in comparison to sporadic MRSA.

The aim of this study was to compare the ability of epidemic versus sporadic MRSA strains to survive on human skin. First, human epidermal models based on N/TERT keratinocytes were exposed to 27 epidemic and 31 sporadic MRSA strains. Viable bacteria were quantified and the expression of 5 host immune factors and 43 bacterial virulence factors was determined. Bacterial presence in the culture supernatants was determined to assess the ability of the strains to breach the epidermal barrier function. Finally, epidermal models based on keratinocytes from different human donors were exposed to five epidemic and seven sporadic MRSA strains to explore potential skin donor variation.

Methods

MRSA strains

MRSA strains originated from the MACOTRA strain collection as assembled by the MACOTRA study group to investigate transmission success of MRSA ¹¹. This collection contains clinical MRSA strains of British, French and Dutch origin, labelled as epidemic or sporadic based on country-specific definitions. In total, 27 epidemic and 31 sporadic MRSA strains of 8 different genetic lineages were included in

this study, comprising 16 representatives from France, 26 from the Netherlands and 16 from the UK (Supplementary Table S1). Twelve strains were selected to study the production of bacterial virulence factors and for additional experiments on Leiden epidermal models (LEMs). For each country, four strains were included in this subset, consisting of five epidemic and seven sporadic representatives (Supplementary Table S1). For each batch of epidermal models, MRSA strains LUH14616 (ST247; NCCB100829) and LUH15091 (ST121) were included as bacterial growth controls with PBS as negative control^{12,13}. Strains were cultured on Trypticase Soy Agar supplemented with 5% sheep blood (Becton Dickinson, Vianen, The Netherlands) at 37°C overnight.

Epidermal model construction

N/TERT-based epidermal models (NEMs) were constructed as described with minor modifications¹⁴. In short, human keratinocytes of the N/TERT cell line (Harvard Medical School¹⁵) were cultured to 80% confluency in Keratinocyte Serum Free Medium with L-glutamine (Gibco, Paisley, Scotland) supplemented with 25 µg/ml bovine pituitary extract, 0.2 ng/ml recombinant human epidermal growth factor 1-53, 0.3 mM CaCl₂ and penicillin-streptomycin at 37°C and 5% CO₂. To construct epidermal models, 2x10⁵ N/TERT keratinocytes in DermaLife K Keratinocyte Complete Medium with LIFE factors (LifeLine Cell Technology, Frederick, USA) were seeded onto cell culture filter inserts with 0.4 µm pores (ThinCerts; Greiner Bio-One, Frickenhausen, Germany) in 12-well plates. The cells were cultured 3 days until confluency followed by replacement of the apical and basal medium for DMEM/Ham's F-12/CnT-Prime 3D barrier medium in a 3:1:4 ratio supplemented with 0.1 µg/ml hydrocortisone, 0.125 µg/ml isoprotenerol, 0.25 µg/ml bovine insulin, 16.5 pM selenious acid, 5 mM L-serine, 5 µM L-carnitine, 1.6 mg/ml BSA, 25 µM palmitic acid, 15 µM linoleic acid, and 7 µM arachidonic acid. The apical medium was removed after 24h, thereby leaving the top cell layers air-exposed and allowing epidermal differentiation. The basal medium was replaced after 3 days of air exposure to the same medium with an increased concentration of linoleic acid (30 µM). This medium was refreshed every 2 days. After 10 days of air-exposure, NEMs were fully differentiated and ready for experimentation.

Leiden epidermal models (LEMs) were based on primary keratinocytes originating from 3 different donors. For our study, fresh plastic surgery surplus skin samples were collected from patients after informed consent. This procedure is in accordance with the Dutch Law and additional approval from ethics committee was not required. According to Article 467 of the Dutch Law on Medical Treatment Agreement and the Code for proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies, coded anonymous surplus tissue can be used for biomedical research when the donor has no objection¹⁶. The Helsinki Declaration principles were followed while working with human tissue.

For keratinocyte isolation, the epidermis of fresh surplus skin was mechanically separated from the dermis and digested to obtain a keratinocyte cell suspension. Next, keratinocytes were cultured in Keratinocyte medium, comprising of DMEM and Ham's F-12 medium at 3:1 ratio supplemented with 5% fetal bovine serum, 0.5 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin, 100 U/ml penicillin and 100 µg/ml streptomycin. Keratinocyte medium was switched to DermaLife with 100 U/ml penicillin and 100 µg/ml streptomycin before the construction of the 3D models. After 24h, 2x10⁵ keratinocytes were seeded onto filter inserts (0.4 µm Costar inserts) in 24-wells plates in DermaLife medium. After 3 days, keratinocytes were

air-exposed by removing the apical medium. The basal medium was replaced with CnT-02-3D medium and keratinocyte medium supplemented with 240 nM BSA, 25 μ M palmitic acid, 15 μ M linoleic acid, and 7 μ M arachidonic acid. Experiments were performed using 10 days air-exposed cultures.

Epidermal model colonization

Before bacterial inoculation, the model medium was replaced by fresh medium without antibiotics. After 24h, models were inoculated with 1×10^5 CFU/ml log phase MRSA in PBS. After 1h at 37°C in 5% CO₂, non-adherent bacteria were plated and counted. At 24h, 48h and 8 days (NEMs) after inoculation, the subnatant, the cell culture medium beneath the air-exposed keratinocyte layer, was screened for bacterial contamination, the models were washed, and the non-adherent bacterial counts were determined. The models were homogenized using a Precellys 24 tissue homogenizer (3x10s at 5,000 rpm, Bertin Technologies, Montigny-le Bretonneux, France) and adherent bacterial counts determined. The lower detection limit for adherent bacteria was 40 CFU/model. For each strain, six replicates of NEMs were prepared, and LUH14616 and LUH15091 served as growth controls; NEMs were incubated with PBS as negative controls.

Bacterial counts on LEMs after 24h and 48h were evaluated for 12 strains in duplicate as described above. Due to limited donor material, each strain was tested on epidermal models of two different donors. LUH14616 was included as a growth control on models of all three donors, and incubated with PBS as negative controls.

Production of antimicrobial peptides, cytokines and chemokines by keratinocytes

NEM subnatants were collected at 24h and 48h after the start of the infection. Duplicates were pooled and subsequently stored at -20°C until further analysis. The levels of antimicrobial peptides human β -defensin (hBD)-2 (LSBIO) and RNase 7 (DS Develop), cytokines interleukin-1 β and interleukin-6 and chemokine interleukin-8 (CXCL-8) (all Biolegend) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The lower limits of detection of hBD-2, RNase 7, IL-1 β , IL-6 and IL-8 were 12 pg/ml, 0.61 ng/ml, 0.5 pg/ml, 15.6 pg/ml and 7.8 pg/ml, respectively.

Bacterial virulence factor expression

The presence of 43 bacterial proteins was detected by a multiplex competitive Luminex assay as described, with slight modifications^{13,17}. Proteins of interest were covalently coupled to the beads as previously described¹⁸⁻²⁰. The tested proteins include (pore-forming) toxins, exfoliative toxins, immune evasion proteins, staphylococcal enterotoxins, staphylococcal superantigen-like proteins, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and others. An overview of included proteins is listed in Supplementary Table S2.

For a subset of 12 MRSA strains, five three-fold serial dilutions of pooled NEM subnatant duplicates, collected at 24h, were made in phosphate buffered saline (PBS) supplemented with 1% (w/v) bovine serum albumin (Roche Diagnostics, Almere, Nederland) and 0.05% (w/v) sodium azide (Sigma-Aldrich, Zwijndrecht, Nederland). These were incubated in equal volumes with a 1:50 dilution of polyclonal human IgG (Sigma-Aldrich) for 35 min at 25°C with continuous shaking at

800 rpm. Incubated subnatant was added in equal volumes to a prepared black flat-bottomed 96-well plate containing 50 μ l of bead mixture. Mean Fluorescent Intensity (MFI) of IgG-bound beads was measured using a multiplex bead-based flow cytometry (Bio-Plex 200, Bio-Rad, Lunteren, Nederland). An uncoupled bead was used as an internal negative control. As experimental controls, subnatants of bacterial growth controls and PBS-treated models were included in the analysis. To control for bacterial interaction with cell culture medium and filter inserts, a separate experiment was carried out where bacteria were inoculated directly onto the cell culture filter without keratinocytes, inserted into the same basal medium as described above for NEMs.

MFI values were plotted to evaluate the levels of captured IgG throughout the subnatant dilution series. Decrease of MFI values was most pronounced in the first three-fold dilution, which was selected for further analysis. Proteins with values < 100 MFI were excluded for data analysis. For the remaining proteins, the relative decrease of MFI in comparison to the untreated control (set at 100% MFI) was calculated. This relative MFI decrease forms a semi-quantitative measure which reflects the presence of the bacterial virulence factor in the subnatant. For each combination of strain and bacterial protein, the difference in relative MFI decrease was calculated between models with and without keratinocytes. If this difference was >20, protein expression was attributed as due to interaction with keratinocytes. A relevant level of protein expression was determined at a cut-off value of 25% in relative MFI decrease.

Statistical analysis

Before statistical testing, descriptive statistics, histograms and QQ plots were evaluated. Results of non-normal distributed data are given as median and range. Statistical analyses were performed using non-parametric tests, i.e., Wilcoxon's rank sum test for independent samples, Wilcoxon signed rank test to account for paired samples or Kruskal Wallis rank sum test for multiple groups. Statistical significance was set at $\alpha = 0.05$, and all analyses were carried out using R *stats* and *pastecs* packages^{21,22}. All figures were made using the *ggplot2* and *patchwork* packages^{23,24}.

Data availability statement

The authors declare that all relevant data of this study are available within the article or from the corresponding author on reasonable request.

Results

Bacterial survival on N/TERT epidermal models (NEMs)

To compare the ability of 27 epidemic and 31 sporadic MRSA strains to survive on human skin, NEMs were inoculated, and bacterial counts of viable adherent and non-adherent MRSA were determined after 24h, 48h and 8 days. Median log CFU/NEM at inoculation was 5.72 (4.00-6.10). Bacterial numbers increased up to 48h after inoculation (8.60 log CFU/NEM (6.02-9.31)) and subsequently decreased to 7.77 log CFU/NEM (6.02-8.83). No difference in counts on NEMs was found between epidemic and sporadic strains for 8 days. For example, the median log CFU/NEM was 7.80 (6.10-8.83) for epidemic strains, and 7.71 (6.02-8.59) for sporadic strains at 8 days ($W = 6421.5$; $p = 0.29$) (Figure 1).

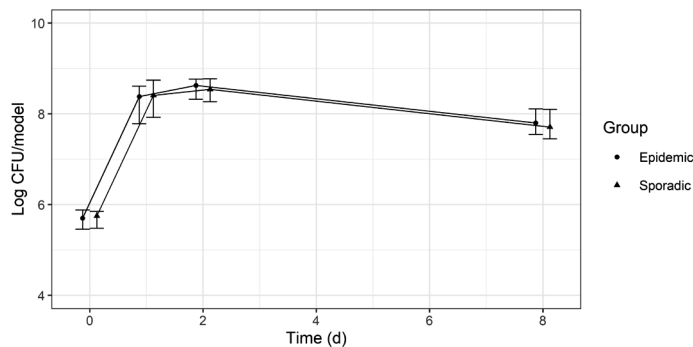


Figure 1. Course of bacterial counts on N/TERT epidermal models.

Bacterial counts for 27 epidemic and 31 sporadic MRSA strains on NEMs were determined at 24h, 48h and 8 days after inoculation of the models; each strain was tested in duplicate. Median log CFU/NEM is shown for epidemic and sporadic strains, with error bars depicting upper and lower quartiles.

Bacterial survival on epidermal models from 3 different human donors

To explore potential differences in survival due to host variation, five epidemic and seven sporadic MRSA strains were tested on Leiden epidermal models (LEMs) based on primary keratinocytes from three donors. Inoculation numbers were comparable for LEMs of the three different donors (5.69 log CFU/NEM (5.37-6.00)). These numbers increased to an overall 7.11 log CFU/LEM (4.46-8.37) over 48h. Sporadic strains showed slightly lower survival numbers compared to epidemic strains at 24h and 48h on LEMs of all donors (Figure 2). These small differences were significant ($W = 1192$, $p = 0.03$ at 24 h; $W = 1400$, $p = 0.003$ at 48 h). Differences in bacterial counts for sporadic versus epidemic strains were most pronounced on LEMs of donor 2 at 48h ($W = 109$, $p = 0.02$).

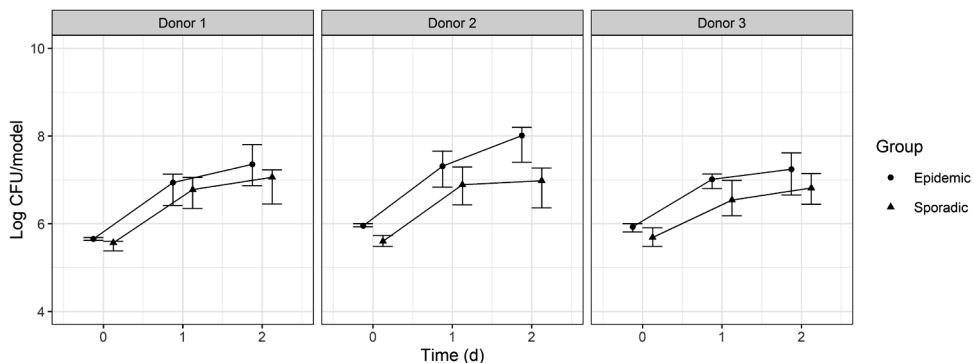


Figure 2. Bacterial counts on Leiden epidermal models (LEMs) from 3 different human donors.

Bacterial counts for 5 epidemic and 7 sporadic MRSA strains on LEMs of 3 different donors were assessed after 24 h and 48 h. Each strain was tested on LEMs of 2 different donors (due to limited supply), in duplicate. Four epidemic strains and six sporadic strains were tested on LEMs from donor 1. Three epidemic and four sporadic strains were tested on LEMs from donor 2. Three epidemic and four sporadic strains were tested on LEMs from donor 3. Median log CFU/NEM is shown for epidemic and sporadic strains on each donor, with error bars depicting upper and lower quartiles.

Production of inflammatory cytokines/chemokines and antimicrobial peptides by N/TERT epidermal models in response to epidemic and sporadic MRSA

To study the response of N/TERT epidermal models to bacterial exposure, the levels of cytokines IL-1 β and IL-6 and chemokine IL-8 and of the antimicrobial peptides hBD-2 and RNase 7 in NEM subnatants were determined for all 58 strains at 24h and 48h after inoculation of the models. Median values of hBD-2 ($V = 90$, $p < 0.001$), RNase 7 ($V = 286$, $p > 0.05$), IL-1 β ($V = 749$, $p > 0.05$) and IL-8 (CXCL-8) ($V = 413$, $p < 0.001$) increased during infection, but not for IL-6 (Figure 3). No differences were found in production of these immune factors between NEMs upon exposure to epidemic or sporadic MRSA strains.

Production of immune modulators, toxins and other proteins by sporadic and epidemic MRSA strains during infection of N/TERT epidermal models

For a subset of 12 strains, the bacterial response upon exposure to N/TERT cells was studied by determining the presence of 43 bacterial proteins in NEM subnatants at 24h after inoculation by multiplex competitive Luminex assay. For 11 of 12 MRSA strains, relevant protein expression attributed to interaction with keratinocytes was found (Table 1). Autolysin Atl2 (11 of 12 strains) was most expressed, followed by gamma-hemolysin B (HlgB; 6/12), alpha-toxin (Hla; 5/12), leukocidins LukD (4/12), LukE (2/12) and LukS (2/12), autolysin Aly (2/12), extracellular adherence protein Eap (2/12) and lipase (2/12). For thermonuclease (nuc), staphylococcal enterotoxin-like O (SEO), staphylococcal superantigen-like protein 5 (SSL5), SSL9 and toxic shock syndrome toxin-1 (TSST-1) expression was found in one strain with Aly, Eap, Hla, LukD, LukE, and LukS expression restricted to epidemic strains only; HlgB was expressed in all 5 epidemic strains and 1 sporadic strain. Due to the low MFI values, data for bacterial proteins Esx-1-associated factor EsxB, Staphylococcal enterotoxin E (SEE) and hypothetical protein SA2097 were excluded from the analysis.

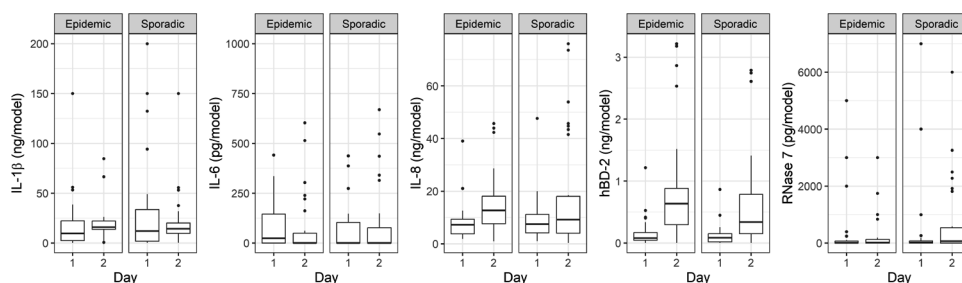


Figure 3. Production of inflammatory cytokines/chemokines and antimicrobial peptides by N/TERT epidermal models in response to epidemic and sporadic MRSA.

IL-1 β , IL-6, chemokine IL-8 and antimicrobial peptides hBD-2 and RNase 7 production by keratinocytes at 1 and 2 days after inoculation with 27 epidemic and 31 sporadic MRSA strains. Boxplots (in the style of Tukey) show median values with upper and lower hinges corresponding to the first and third quartiles and whiskers representing $\pm 1.5 \times \text{IQR}$.

Strain	Genetic lineage	Infection or carriage	Epidemic or sporadic	Filter passage at 24h	Aly ¹	Atl2 ¹	Eap ¹	Hla ¹	HlgB ¹	Lipase ¹	LukD ¹	LukE ¹	LukS ¹	Nuc ¹	SEO ¹	SSL5 ¹	SSL9 ¹	TSST-1 ¹
11002	CC8	Carriage	Epidemic	0%		93,7		86,9	61,0		42,8		88,5					
11016	CC5	Carriage	Epidemic	100%	94,8	94,9		71,4	74,6	89,2	35,6							
11127	CC22	Infection	Epidemic	0%		95,5	38,5	82,1	56,9									
11257	CC5	Infection	Epidemic	50%		96,4	50,7	93,2	90,8		81,5	68,3						
11259	CC8	Infection	Epidemic	100%	82,5	97,0		85,9	88,8		61,7	46,2	44,5					
11051	CC5	Infection	Sporadic	NA		84,7												
11086	CC8	Infection	Sporadic	0%		94,2												
11107	CC30	Infection	Sporadic	100%		95,6		42,9	82,5				93,3					25,4
11112	CC30	Infection	Sporadic	0%														
11122	CC22	Infection	Sporadic	50%		74,1												
11193	CC8	Infection	Sporadic	50%		93,7									71,4	61,0	20,3	
11210	CC5	Infection	Sporadic	0%		71,0												

Table 1. Presence of bacterial virulence factors in N/TERT epidermal model subnatants at 24h after inoculation

Presence of bacterial virulence factors in NEM subnatants after inoculation with epidemic (n=5) and sporadic (n=7) MRSA as determined by a multiplex competitive Luminex assay. Passage through the keratinocyte cell layers and filter insert below was observed at 24h, 0%: no passage; 50%: passage in a single NEM; 100%: passage in both NEMs. (1): Data are given as inverted (x -1) percentage of the untreated control; higher MFI values represent higher levels of virulence factors present. Values were considered relevant if the MFI values were > 25% compared to the untreated control. Blanks indicate no relevant values. Presence of virulence genes is given as P: present, or A: absent

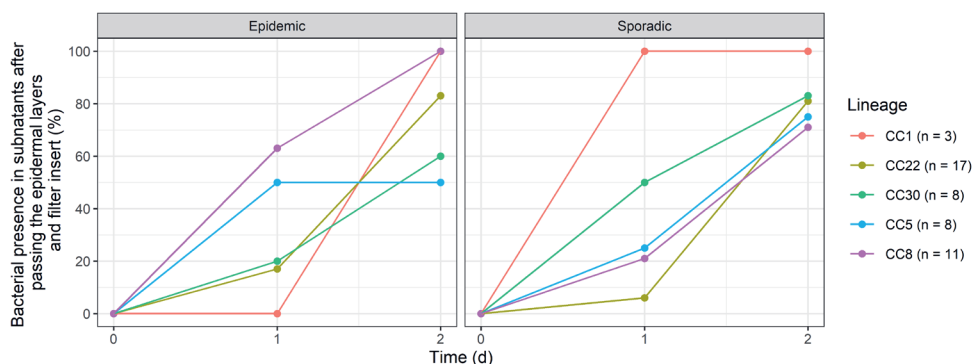


Figure 4. MRSA within the culture subnatants upon interaction with NEM keratinocytes.

Bacterial presence in the subnatant indicate that bacteria have passed through the keratinocyte cell layers and filter below. Results are available for 27 epidemic and 31 sporadic strains and shown as percentage of models demonstrating penetration of epidermal layers. Subnatant bacterial presence increased from 27% at 24h to 78% at 48h and 100% at day 8.

Passage of bacteria through the N/TERT epidermal models during infection

We observed an occasional presence of bacteria in the culture medium indicating their passage through the keratinocyte cell layers and the filter insert below. A control experiment showed that strain LUH14616 did not penetrate the filter insert when keratinocytes were absent, indicating that their interaction facilitated passage. Bacteria were found in 27% of the model subnatants at 24 h, in 78% at 48h and in 100% at 8 days. Subnatant contamination within 24 h was most frequent in epidemic strains of CC8 (63% of models, 3 of 4 strains) and CC5 (50%, 4/6) and in one sporadic CC1 strain as opposed to epidemic strains of CC22 (17%, 2/9) and CC30 (20%, 1/5) (Figure 4). No association was evident between keratinocyte layer disruption at 24h and the expression of the studied virulence factors.

Discussion

This study explored the ability of 58 MRSA strains to survive on human epidermal models in relation to their epidemic or sporadic behavior. Our data revealed a small but significant difference in bacterial counts between sporadic and epidemic strains on 3D epidermal models based on primary keratinocytes. Here, models exposed to sporadic strains showed lower bacterial counts than those exposed to epidemic strains. However, comparable counts were found for sporadic and epidemic strains on 3D N/TERT-based models. These data indicate that the outcome of interactions between MRSA strains and human skin may differ among donors. Potential factors leading to reduced skin colonization by *S. aureus* are nutrient competition, production of antimicrobial peptides, sebaceous lipids and free fatty acids, and host-specific immunity^{25,26}. In this regard, we found significantly increased production of immune factors hBD-2 and IL-8 by NEMs after inoculation, which confirms the development of an innate immune response in our models upon colonization by *S. aureus*. However, the expression of these immunological mediators by the models did not differ between sporadic and epidemic strains. Furthermore, interaction with N/TERT cells induced the expression

of various pore-forming toxins, extracellular adherence proteins, immune evasion proteins and autolysins by epidemic strains primarily. Various studies have suggested increased skin colonization by epidemic MRSA due to the expression of virulence factors ^{8,27}. However, expression of toxins had no effect on bacterial survival in our study. In conclusion, these data suggest that both bacterial and human factors influence the complex interaction between pathogen and host and determines the fate of a *S. aureus* strain when exposed to human skin.

In our study, we found that epidemic, but not sporadic, MRSA strains expressed pore-forming toxins, including alpha-hemolysin (Hla), gamma-hemolysin (HlgB), PVL (LukS) and LukED, upon interaction with NEMs. Upregulation of *hla*, *hlgB*, *lukS-PV*, *LukF-PV*, *lukE* and *lukD* genes by *S. aureus* strains has been shown earlier in skin explants and in drainage material of cutaneous abscesses, which strengthens our findings ^{28–32}. All these toxins exhibit cytotoxic activity towards leukocytes, i.e., monocytes and neutrophils ³³. LukED and Hla are also cytotoxic to dendritic cells and keratinocytes, respectively ^{33,34}, which highlights the role of these toxins in bacterial invasion of the skin. Moreover, we observed passage of *S. aureus* through the cell culture filter, which was mediated by the interaction between the bacteria and keratinocytes. Most likely, factors produced by skin cells and/or bacterial cells facilitated the destruction of the filter. Passage of the filter was observed for all genetic lineages but occurred more quickly for epidemic strains of CC8 and CC5. Despite these observations, we were unable to show an association between keratinocyte damage and the expression of bacterial toxins.

Next to toxin production, we found expression of the major autolysin Atl, which plays a dominant role in peptidoglycan metabolism, and was therefore expected for all strains ^{35,36}. Additionally, Atl contributes to surface attachment, biofilm formation and internalization into host cells ^{35,36}. Extracellular adherence protein (Eap) was also expressed. Besides its role in immune evasion, Eap affects the interaction between *S. aureus* and keratinocytes through interference with keratinocyte proliferation and migration, wound healing and stimulation of bacterial adherence and internalization ^{37–40}.

Earlier studies have shown higher expression levels of certain virulence factors in epidemic MRSA compared to sporadic MRSA. Higher expression of *hla* was found for USA300 compared to USA400 in a rat pneumonia model ⁷. Hla expression was also higher in community-associated MRSA compared to hospital-associated MRSA ⁴¹. Epidemic MRSA strain MW2 induced more cytotoxicity in human primary keratinocytes when Hlb was expressed ²⁷. Interestingly, despite our small sample size for virulence factor experiments, we found that toxin production was mostly limited to epidemic strains. Taken together, these results might imply that epidemic and sporadic strains are equally capable to survive on the epidermis, but epidemic strains might be more capable of responding to immune cells upon infection than sporadic strains.

To our knowledge, this is the first study that compares the ability of large numbers of clinical epidemic and sporadic MRSA strains of different genetic backgrounds to survive on human skin. It should be noted that the definition of epidemic and sporadic strains differed between originating countries due to different testing and sampling practices. This could have affected our results. As our models consisted of an epidermis only, another limitation was the absence of immune cells which normally reside in the dermis. For future work, skin models consisting of dermis

and epidermis would be preferred to gain more insight into the innate immune response against natural MRSA infection.

Overall, we found that epidemic and sporadic MRSA strains were equally capable of surviving on epidermal models of the N/TERT cell line. Nevertheless, survival of epidemic strains was more successful than sporadic strains on epidermal models of primary keratinocytes, indicating a possible role for both host and bacterial factors in MRSA survival and transmission. Additionally, various pore-forming toxins, including alpha-hemolysin, PVL and LukED, were exclusively expressed by epidemic strains, giving these strains a benefit when encountering neutrophils. Together, these findings provide potential explanations for the transmission success of epidemic MRSA strains.

Acknowledgements

We are grateful to all members of the MACOTRA study group for their input. The MACOTRA study group consists of Valérie O. Baede ¹, Anaïs Barray ^{2,3}, Sake J. de Vlas ⁴, Anneke S. de Vos ⁴, Arya Gupta ⁵, Antoni P. A. Hendrickx ⁶, Gwenan M. Knight ⁷, Mirjam E. E. Kretzschmar ^{6,8}, Gérard Lina ^{2,3}, Jodi A. Lindsay ⁵, Jean-Philippe Rasigade ^{2,3}, Leo Schouls ⁶, Mehri Tavakol ¹, Margreet C. Vos ¹, Willem J. B. van Wamel ¹, Adam A. Witney ⁵

References

1. Wertheim, H. F. *et al.* The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* **5**, 751–762 (2005).
2. DeLeo, F. R., Otto, M., Kreiswirth, B. N. & Chambers, H. F. Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* **375**, 1557–1568 (2010).
3. Enright, M. C. *et al.* The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci.* **99**, 7687–7692 (2002).
4. Stefani, S. *et al.* Methicillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *Int. J. Antimicrob. Agents* **39**, 273–282 (2012).
5. Ellington, M. J. *et al.* Decline of EMRSA-16 amongst methicillin-resistant *Staphylococcus aureus* causing bacteraemias in the UK between 2001 and 2007. *J. Antimicrob. Chemother.* **65**, 446–448 (2010).
6. Planet, P. J. Life After USA300: The Rise and Fall of a Superbug. *J. Infect. Dis.* **215**, S71–S77 (2017).
7. Montgomery, C. P. *et al.* Comparison of Virulence in Community-Associated Methicillin-Resistant *Staphylococcus aureus* Pulsotypes USA300 and USA400 in a Rat Model of Pneumonia. *J. Infect. Dis.* **198**, 561–570 (2008).
8. Planet, P. J. *et al.* Emergence of the Epidemic Methicillin-Resistant *Staphylococcus aureus* Strain USA300 Coincides with Horizontal Transfer of the Arginine Catabolic Mobile Element and speG - mediated Adaptations for Survival on Skin. *MBio* **4**, e00889-13 (2013).
9. Glaser, P. *et al.* Demography and Intercontinental Spread of the USA300 Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Lineage. *MBio* **7**, e02183-15 (2016).
10. O’Gara, J. P. Into the storm: Chasing the opportunistic pathogen *Staphylococcus aureus* from skin colonisation to life-threatening infections. *Environ. Microbiol.* **19**, 3823–3833 (2017).
11. MACOTRA Consortium. Markers of epidemiological success in European MRSA populations - Details of isolate selection. (2022) doi:10.24376/rd.sgul.19070333.v1.
12. Croes, S. *et al.* *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. *BMC Microbiol.* **9**, 229 (2009).
13. den Reijer, P. M. *et al.* Detection of Alpha-Toxin and Other Virulence Factors in Biofilms of *Staphylococcus aureus* on Polystyrene and a Human Epidermal Model. *PLoS One* **11**, e0145722 (2016).
14. Wu, K., Conly, J., McClure, J.-A., Kurwa, H. A. & Zhang, K. Arginine Catabolic Mobile Element in Evolution and Pathogenicity of the Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strain USA300. *Microorganisms* **8**, 275 (2020).
15. van Drongelen, V. *et al.* Barrier Properties of an N/TERT-Based Human Skin Equivalent. *Tissue Eng. Part A* **20**, 3041–3049 (2014).
16. Dutch Central Committee on Research Involving Human Subjects. (<https://english.ccmo.nl/investigators/additional-requirements-for-certain-types-of-research/non-wmo-research/research-with-human-tissue>). Accessed 2 November 2022.
17. Hansenová Maňásková, S., Bikker, F. J., Veerman, E. C. I., van Belkum, A. & van Wamel, W. J. B. Rapid detection and semi-quantification of IgG-accessible *Staphylococcus aureus* surface-associated antigens using a multiplex competitive Luminex assay. *J. Immunol. Methods* **397**, 18–27 (2013).
18. den Reijer, P. M. *et al.* Characterization of the Humoral Immune Response during *Staphylococcus aureus* Bacteremia and Global Gene Expression by *Staphylococcus aureus* in Human Blood. *PLoS One* **8**, e53391 (2013).

19. Verkaik, N., Brouwer, E., Hooijkaas, H., van Belkum, A. & van Wamel, W. Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins. *J. Immunol. Methods* 335, 121–125 (2008).
20. Martins, T. B., Augustine, N. H. & Hill, H. R. Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to group A streptococci. *J. Immunol. Methods* 316, 97–106 (2006).
21. Grosjean, P. & Ibanez, F. pastecs: Package for Analysis of Space-Time Ecological Series. (2018).
22. R Core Team. R: A language and environment for statistical computing. (2017).
23. Pedersen, T. L. patchwork: The Composer of Plots. (2020).
24. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. (2016).
25. Krismer, B., Weidenmaier, C., Zipperer, A. & Peschel, A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nat. Rev. Microbiol.* 15, 675–687 (2017).
26. Kwiecien, K. et al. Architecture of antimicrobial skin defense. *Cytokine Growth Factor Rev.* 49, 70–84 (2019).
27. Katayama, Y., Baba, T., Sekine, M., Fukuda, M. & Hiramatsu, K. Beta-Hemolysin Promotes Skin Colonization by *Staphylococcus aureus*. *J. Bacteriol.* 195, 1194–1203 (2013).
28. Loughman, J. A., Fritz, S. A., Storch, G. A. & Hunstad, D. A. Virulence gene expression in human community-acquired *Staphylococcus aureus* infection. *J. Infect. Dis.* 199, 294–301 (2009).
29. Date, S. V. et al. Global Gene Expression of Methicillin-resistant *Staphylococcus aureus* USA300 During Human and Mouse Infection. *J. Infect. Dis.* 209, 1542–1550 (2014).
30. Burian, M. et al. Adaptation of *Staphylococcus aureus* to the Human Skin Environment Identified Using an ex vivo Tissue Model. *Front. Microbiol.* 12, 728989 (2021).
31. Cruz, A. R., van Strijp, J. A. G., Bagnoli, F. & Manetti, A. G. O. Virulence Gene Expression of *Staphylococcus aureus* in Human Skin. *Front. Microbiol.* 12, 692023 (2021).
32. Pulia, M. S. et al. Expression of Staphylococcal Virulence Genes In Situ in Human Skin and Soft Tissue Infections. *Antibiotics* 11, 527 (2022).
33. Seilie, E. S. & Bubeck Wardenburg, J. *Staphylococcus aureus* pore-forming toxins: The interface of pathogen and host complexity. *Semin. Cell Dev. Biol.* 72, 101–116 (2017).
34. Grumann, D., Nübel, U. & Bröker, B. M. *Staphylococcus aureus* toxins – Their functions and genetics. *Infect. Genet. Evol.* 21, 583–592 (2014).
35. Houston, P., Rowe, S. E., Pozzi, C., Waters, E. M. & O’Gara, J. P. Essential Role for the Major Autolysin in the Fibronectin-Binding Protein-Mediated *Staphylococcus aureus* Biofilm Phenotype. *Infect. Immun.* 79, 1153–1165 (2011).
36. Hirschhausen, N. et al. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cell. Microbiol.* 12, 1746–1764 (2010).
37. Woehl, J. L. et al. The Extracellular Adherence Protein from *Staphylococcus aureus* Inhibits the Classical and Lectin Pathways of Complement by Blocking Formation of the C3 Proconvertase. *J. Immunol.* 193, 6161–6171 (2014).
38. Eisenbeis, J. et al. The extracellular adherence protein (Eap) of *Staphylococcus aureus* acts as a proliferation and migration repressing factor that alters the cell morphology of keratinocytes. *Int. J. Med. Microbiol.* 307, 116–125 (2017).
39. Joost, I. et al. Transcription Analysis of the Extracellular Adherence Protein from *Staphylococcus aureus* in Authentic Human Infection and In Vitro. *J. Infect. Dis.* 199, 1471–1478 (2009).

40. Bur, S., Preissner, K. T., Herrmann, M. & Bischoff, M. The *Staphylococcus aureus* Extracellular Adherence Protein Promotes Bacterial Internalization by Keratinocytes Independent of Fibronectin-Binding Proteins. J. Invest. Dermatol. 133, 2004–2012 (2013).
41. Tavares, A. et al. Insights into Alpha-Hemolysin (Hla) Evolution and Expression among *Staphylococcus aureus* Clones with Hospital and Community Origin. PLoS One 9, e98634 (2014).

Supplementary material

Supplementary Table S1. Details of MRSA strains included in the study

Supplementary Table S2. Luminex proteins included in the study

Chapter 6

Nasal microbiome disruption and recovery after mupirocin treatment in *Staphylococcus aureus* carriers and noncarriers

Valérie O. Baede[#], Anaïs Barray[#], Mehri Tavakol, Gérard Lina, Margreet C. Vos^{*}, Jean-Philippe Rasigade^{*}

^{#,*} These authors contributed equally

Based on:

Scientific Reports. **12**, 19738 (2022)

DOI: [10.1038/s41598-022-21453-4](https://doi.org/10.1038/s41598-022-21453-4)

Abstract

Nasal decolonisation procedures against the opportunistic pathogen *Staphylococcus aureus* rely on topical antimicrobial drug usage, whose impact on the nasal microbiota is poorly understood. We examined this impact in healthy *S. aureus* carriers and noncarriers.

This is a prospective interventional cohort study of 8 *S. aureus* carriers and 8 non-carriers treated with nasal mupirocin and chlorhexidine baths. Sequential nasal swabs were taken over 6 months. *S. aureus* was detected by quantitative culture and genotyped using *spa* typing. RNA-based 16S species-level metabarcoding was used to assess the living microbial diversity.

The species *Dolosigranulum pigrum*, *Moraxella nonliquefaciens* and *Corynebacterium propinquum* correlated negatively with *S. aureus* carriage. Mupirocin treatment effectively eliminated *S. aureus*, *D. pigrum* and *M. nonliquefaciens*, but not corynebacteria. *S. aureus* recolonisation in carriers occurred more rapidly than recolonisation by the dominant species in noncarriers (median 3 vs. 6 months, respectively). Most recolonising *S. aureus* isolates had the same *spa* type as the initial isolate.

The impact of mupirocin-chlorhexidine treatment on the nasal microbiota was still detectable after 6 months. *S. aureus* recolonisation predated microbiota recovery, emphasizing the strong adaptation of this pathogen to the nasal niche and the transient efficacy of the decolonisation procedure.

Introduction

Staphylococcus aureus is an opportunistic pathogen and a frequent cause of severe infections. Approximately 20% of the general population are persistent *S. aureus* carriers and another 30% are intermittent carriers ¹. *S. aureus* is commonly carried in the nose and less frequently in the throat, skin, and perineum ¹.

S. aureus carriers are at higher risk of infection after invasive procedures and surgery ^{2,3}. To prevent infections, several countries recommend eliminating *S. aureus* from the nose prior to the at-risk intervention using a decolonisation procedure ⁴. This typically involves topical antimicrobial treatment with mupirocin nasal ointment with or without chlorhexidine cutaneous body and hair wash. Different decolonisation approaches have emerged due to costs and organisational issues in health care ⁵. While some advise to treat all patients undergoing at-risk interventions, others limit decolonisation to confirmed carriers only.

While we know that the nasal microbiome composition is related to *S. aureus* presence ^{6,7}, the impact of decolonisation procedures on the nasal microbiota is not yet fully understood. In previous nasal microbiome studies, *S. aureus* carriage was associated with higher relative abundances of *Cutibacterium acnes*, *Corynebacterium accolens* and non-aureus staphylococci, and with lower abundances of *Corynebacterium pseudodiphtheriticum*, *Dolosigranulum* spp and *Cutibacterium granulosum* ^{6,7}. These associations suggest that the distribution of microbial species in the nose influences *S. aureus* persistence, possibly through competition for nutrients and epithelial binding sites ⁸. In turn, the alteration of the microbial distribution after a decolonisation procedure might impact the likelihood of persistent *S. aureus* recolonisation and, from a clinical standpoint, of decolonisation failure. However, the magnitude and duration of microbiota alterations after decolonisation are not elucidated. So far, a single-patient microbiome study found shifts in the composition and biodiversity of the nasal microbiota after mupirocin treatment ⁹, contrasting with a previous culturomics study of 5 healthy volunteers in which no significant change of microbiota richness and diversity were found up to 1 month after decolonisation ¹⁰.

To decipher the relationships between *S. aureus* nasal carriage, the nasal microbiota and decolonisation procedures, we conducted a prospective interventional cohort study of *S. aureus* carriers and noncarriers, monitoring microbial community changes over 6 months after mupirocin-chlorhexidine treatment. Using quantitative cultures and 16S metabarcoding, we examined the impact of decolonisation on bacterial communities and the delay to recolonisation with *S. aureus* and other dominant species.

Methods

Study population and study design

This is a prospective interventional cohort study of healthy *S. aureus* carriers and noncarriers in the Netherlands. All experiments were performed in accordance with the Dutch Medical Research Involving Human Subjects Act (WMO). The study protocol was approved by the local Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam, The Netherlands (MEC-2018-091). Written informed consent was obtained for all participants. Participants were recruited through advertisements at Dutch universities and the research teams social networks. Exclusion criteria were age <18 years, use of antibiotics, antiparasitics,

antifungals or probiotics 3 months prior to recruitment, known allergy to components of the intervention treatment, pregnant and breastfeeding women, known chronic diseases affecting the immune system, severe chronic skin diseases, immunocompromised status, or use of immunosuppressant drugs.

After filling out an eligibility questionnaire, all volunteers were screened for *S. aureus* carriage as described previously ¹¹. *S. aureus* carriage was determined by quantitative culture of 2 weekly nasal swabs. Persistent *S. aureus* carriers were defined as 2 positive cultures with >8 CFU/mL for each culture. Noncarriers were defined as 2 *S. aureus*-negative cultures. Intermittent *S. aureus* carriers were excluded from further participation in the study. Eligible volunteers were enrolled on a first-come, first-served basis.

Eligible participants were asked to fill out a questionnaire regarding risk factors for *S. aureus* acquisition. All participants received decolonisation treatment. Decolonisation consisted of mupirocin nasal ointment (2%, GlaxoSmithKline BV, Zeist, the Netherlands) twice daily and chlorhexidine gluconate cutaneous solution (4%w/v, Regent Medical Overseas Limited, Oldham, UK) once daily, both for 5 days.

Nasal samples were taken 1 day before decolonisation (D0) and 2 days (D7), 1 month (M1), 3 months (M3) and 6 months (M6) after decolonisation. All participants received a personal demonstration for nasal sampling by the executive researcher. Thereafter, all specimens were taken by the participants by inserting a swab (ESwab, 490CE.A, Copan Italia, Brescia, Italy) into one nostril and rotating 5 times, repeating this in the second nostril using the same swab. Swabs were collected in a container filled with 1 ml modified Liquid Amies, a collection and transport solution, and sent through regular mail service (non-temperature controlled) or deposited at the laboratory personally.

***S. aureus* quantitative culture**

Quantitative *S. aureus* cultures were conducted to examine the dynamics of *S. aureus* carriage over the 6-month follow-up period after decolonisation. Swab containers were vortexed for 20s before plating. Serial dilutions of Amies medium were plated onto phenol mannitol salt agar (PHMA) and incubated for 2 days at 37°C. Swabs were placed in phenol mannitol salt broth (PHMB) and incubated for 7 days at 37°C for enrichment. *S. aureus* growth confirmed by a latex agglutination test (Staph Plus Latex Kit, Diamondial, Vienna, Austria). Morphologically different *S. aureus* colonies were selected for *spa* typing and methicillin resistance screening using BBL CHROMagar MRSA II agar (BD, Breda, the Netherlands).

***Spa* typing**

Molecular typing of *S. aureus* isolates was performed to infer whether recolonisation with *S. aureus* in decolonised carriers involved the same *spa*-type. Typing was limited to the last *S. aureus* positive culture moment and the last *S. aureus* positive culture moment after decolonisation in recolonised carriers. *S. aureus* DNA lysates were prepared by boiling in 10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0 or extraction with the QIAamp DNA Mini Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. Amplification of the *S. aureus* protein A (*spa*) repeat region was performed by PCR with 2 sets of primers. One set consisted of forward primer *spa*-1113, 5'-TAAAGACGATCCTTCGGTGAGC-3' and reverse primer *spa*-1514, 5'-CAGCAGTAGTGCCGTTTGCTT-3' ¹². The other

set consisted of forward primers *spa-F1*, 5'- AACAAACGTAACGGCTTCATCC-3' and *spa-F2* 5'- AGACGATCCTTCAGTGAGC-3' and reverse primer *spa-R1* 5'-GCTTTTGCAATGTCATTTACTG-3'. Amplicons were purified with ExoSAP-IT (Applied Biosystems) according to the manufacturer's instructions and sent for sequence analysis (Baseclear, Leiden, the Netherlands). Resulting sequences were analysed using BioNumerics v7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) and the *spa* types were assigned by use of the RidomStaphType database (Ridom GmbH, Würzburg, Germany).

16S ribosomal RNA sequencing of nasal microbiota

The impact of decolonisation on the nasal microbiome and the recovery of the microbiome structure after decolonisation were examined by means of 16S rRNA metabarcoding. Amies medium from each nasal swab container was stored at -80°C on the day of receipt at the study laboratory in Rotterdam, NL, then sent at -80°C to the microbiome analysis laboratory in Lyon, FR. To properly capture the impact of decolonisation on the living microbiota, metabarcoding used RNA-based 16S ribosomal RNA (rRNA, which is preserved in living cells but quickly cleared after cell death or lysis) rather than the DNA coding sequence, as DNA can persist for prolonged time periods after cell death^{13–16}. RNA was extracted using the Mag Bind® Total RNA 96 Kit (Omega Bio-tek) tissue protocol from 150 µL of samples' material. Cell lysis was performed using beads (Disruptor plate C plus – Omega Bio-tek) and proteinase K for 15 min at 2600 rpm, followed by 10 min at room temperature without agitation, and finished with a DNase I digestion of 20 min at room temperature. RNA was quantified using QuantiFluor RNA kit on Tecan Safire (TECAN). 10 ng total RNA was used for reverse transcription using FIREScript RT cDNA synthesis kit (Solis Biodyne) with random primers, then cDNA was purified with SPRIselect reagent (Beckman coulter) and quantified.

The rRNA V1-V3 region was PCR amplified using the 5X HOT BIOAmp® BlendMaster Mix 12,5 mM MgCl₂ (Biofidal), 10X GC rich Enhancer (Biofidal) and BSA 20 mg/ml. The PCR reaction consisted of 30 cycles at 56°C using the forward primer 27F, 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGAGTTTGATCCTGGCTCAG-3' and reverse primer 534R, 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG-3' in 25 µL of solution. PCR products were purified using SPRIselect beads (Beckman Coulter) in 20 µL nuclease-free water and quantified using QuantiFluor dsDNA (Promega). Samples were indexed with Illumina's barcodes with the same PCR reagents during a 12 cycles PCR, then purified and quantified as previously mentioned. Samples were normalised and pooled, then sequenced using Illumina MiSeq V3 Flow Cell following the constructor's recommendations for a 2x300 bp paired-end application. A mean of 130k proofread reads per sample was obtained.

Experiment buffers were used as negative controls to detect contamination by out-of-sample bacterial RNA. RNA extraction was controlled using an in-house mix of live *Staphylococcus aureus* ATCC29213 and *Escherichia coli* ATCC25922 in equal proportions, allowing for assessing extraction bias in Gram-positive and -negative bacteria. PCR amplification bias was controlled using a commercial DNA mix of 8 bacterial species (ZymoBIOMICS™ Microbial Community DNA Standard).

Bioinformatics and statistical analyses

Sequencing reads were quality checked and trimmed. Paired-ended read pairs were merged using BBMap version 38.49 (available at <https://sourceforge.net/>

projects/bbmap/), with default options besides a minimum single size of 150bp with an average Phred quality score higher than 10, and a total pair size of minimum 400bp. PCR adapters were removed with cutadapt v.2.1 (Martin 2011) then dereplicated using vsearch v.2.12.0¹⁷ with the sizeout option. For species assignment, reads were aligned to sequences of NCBI blast 16S_ribosomal_RNA database (version date 03.12.2020) using Blastn v.2.11.0+^{18,19}, keeping a maximum of 20 reference targets. Read counts per bacterial species were normalised to account for taxon-specific variations of the copy number of 16S rRNA genes using NCBI rrnDB-5.5 database based on the mean gene copy number in the taxon.

To optimise the resolution of sequencing read taxonomic assignment, we used in-house bioinformatic software publicly available at <https://github.com/rasigad-elab/taxonresolve>. Briefly, when a read matches sequences from several species with identical alignment scores, taxonomic assignment pipelines typically output the higher taxonomic level such as the genus (e.g., *Staphylococcus* spp. when a read matches *S. aureus* and *S. epidermidis*). This loss of information can be problematic when species-level discrimination is important. To prevent losing species-level information, the *taxonresolve* software assigns reads with uncertain species to groups of species rather than to genera.

Bacterial species deemed present from contaminating sources such as kits reagents and found in negative controls, mostly from the *Bacillus* genera, were removed. A total of 1,376 species or group of species were retained. The rarefaction curves corresponding to the sequencing effort to assess the species richness within samples are shown in Supplementary Figure 1. Most samples reached a plateau after 40 000 sequences.

Given the small sample size compared to the number of variables and species considered in this study, no hypothesis testing was performed, and we provide a descriptive assessment of the results. In figures, 95% confidence intervals of the means were computed based on normal approximation, after log transformation for CFU/mL and log odds transformation for quantities restricted to the [0,1] interval, such as proportions.

In microbial diversity analyses, we retained the 9 most prevalent bacterial species and pooled the other species into an 'Others' category. To assess the disruption and possible recovery of the microbiota, the divergence of sampled microbiota relative to the initial, pre-treatment microbiota (D0) was assessed using the Bray-Curtis dissimilarity at each sampling time point relative to the first sample of the same patient.

Software code of the analyses are available at <https://github.com/rasigad-elab/macotra-metabarcoding>. Data are available at <https://zenodo.org/record/6382657>. Analyses and figures used R software v3.6.0²⁰ with packages dplyr²¹, ggplot2²², vegan²³, and MicrobiomAnalyst available at <https://www.microbiomeanalyst.ca>^{24,25}.

Results

S. aureus elimination and recolonisation

Of 35 volunteers, 8 carriers and 8 noncarriers were included (see flowchart of patient selection in Figure 1). The *S. aureus* carrier group consisted of 3 males and 5 females of 22-71 years old (median, 26 years). Noncarriers were 2 males and 6 females aged 18-62 years (median, 56 years).

No participants reported the use of antivirals, antiparasitics, immunosuppressants or probiotics in the 3 months prior or during the study. One noncarrier reported the use of amoxicillin/clavulanic acid, 5 days prior to the D0 sampling and again between the M3 and M6 sampling. This participant was retained as antimicrobial use occurred after recruitment and the microbiota composition did not differ from other noncarriers pre-decolonisation. No participants reported previous MRSA carriage. All 16 participants had at least 1 risk factor for *S. aureus* acquisition (Supplementary Table 1).

The dynamics of *S. aureus* elimination and recolonisation after the decolonisation treatment were examined using quantitative culture (Figure 2A) and RNA metabarcoding (Figure 2B). Both methods showed a steep decrease in *S. aureus* loads immediately after decolonisation followed by a gradual increase indicating recolonisation for some carriers. Failed decolonisation in one carrier was confirmed

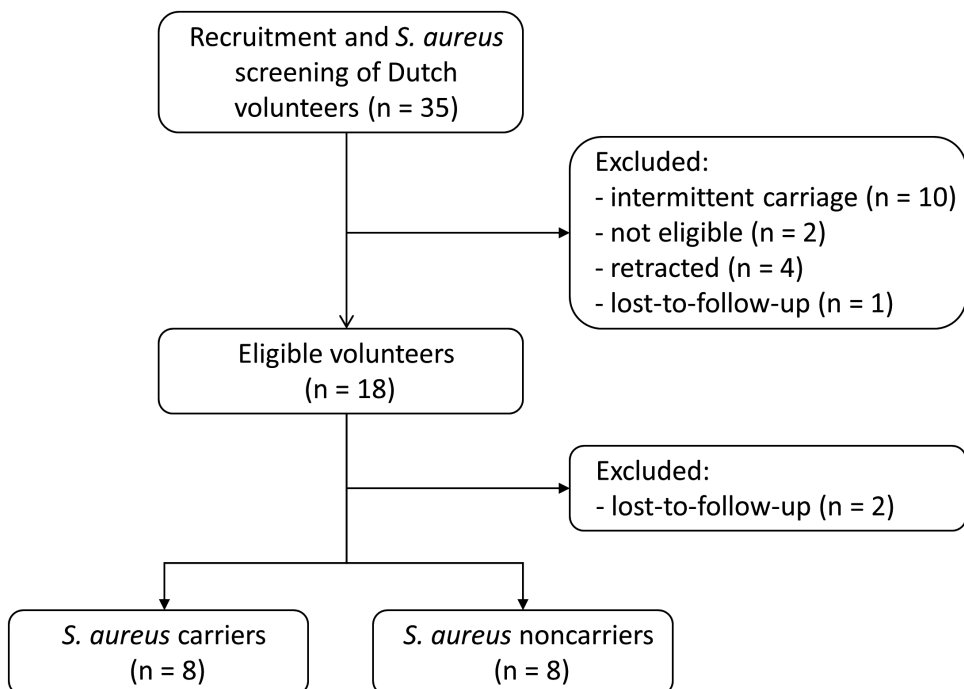


Figure 1. Flowchart of participant recruitment.

In total, 35 volunteers were recruited and screened for eligibility. Sixteen participants completed the study, of which 8 carriers and 8 noncarriers.

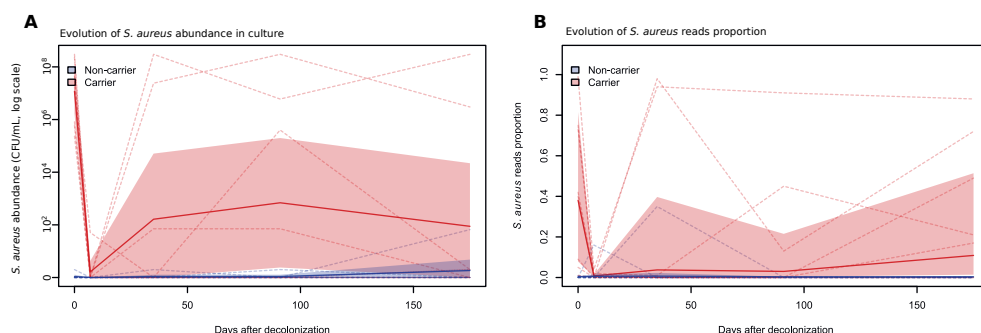


Figure 2. Dynamics of *S. aureus* abundance in nasal samples of carriers and noncarriers undergoing decolonisation.

Shown are *S. aureus* abundance in quantitative culture (CFU/mL on a log scale; A) and proportion in 16S RNA metabarcoding (B) through time in 8 carriers (red) and 8 noncarriers (blue). D0 and D7 denote samples taken immediately before and after the decolonisation procedure, respectively. Dashed lines denote each participant's data. Solid lines and coloured band denote the mean and 95% confidence interval. Both culture and metabarcoding analysis identified a sharp decrease of *S. aureus* abundance after decolonisation followed by recolonisation. On average, post-decolonisation abundance of *S. aureus* was less than before decolonisation.

in the first post-decolonisation sample by both methods (Figure 2). Recolonisation was defined as a *S. aureus* positive culture (>8 CFU/mL) post-decolonisation. Five carriers (C1, C2, C5, C6 and C7) got recolonised during the follow-up period, including 3 carriers within 1 month post-decolonisation. In the noncarrier group, 4 *S. aureus* positive cultures were found post-decolonisation, 3 of which with only 1 CFU/mL.

RNA metabarcoding showed different recolonisation results. While also 5 carriers (C1, C2, C3, C5 and C8) were recolonised according to RNA metabarcoding, discrepancies were found for 4 carriers (C3, C6, C7 and C8). For 2 carriers (C5 and C8), RNA metabarcoding showed recolonisation without a positive culture. Another 2 carriers (C6 and C7) showed no recolonisation in RNA metabarcoding despite a positive culture.

Spa types were determined in carriers exhibiting *S. aureus* recolonisation in culture ($n=5$). All but one recolonised *S. aureus* carriers showed the same *spa* type in pre- and post-decolonisation samples. In 2 carriers, a different *spa* type was found, suggesting transient colonisation by a strain different from the pre-decolonisation carriage strain. *Spa* typing results are shown in Table 1. Details of recolonisation delay and CFU/mL loads are shown in Supplementary Figure 2. No phenotypic resistance to methicillin was found in the tested isolates.

Overall, the *S. aureus* decolonisation remained successful over a 6-month period in only 3 participants (38%) (Figure 2 and Supplementary Figure 2), consistent with previous findings²⁶. Interestingly, the metabarcoding approach detected small proportions (~ 1 -5%) of *S. aureus* reads 2 days and 1 month after decolonisation in several noncarriers (Figure 2B). This might reflect transient invasion of the nasal niche by *S. aureus* isolates, possibly facilitated by the disruption of the nasal microbiome induced by decolonisation, as described in gut microbiota after antibiotic-induced perturbations²⁷. This intermittent carriage is to be expected in the normal population.

Participant	Pre-decolonisation <i>spa</i> -type	Post-decolonisation <i>spa</i> -types (delay)
C1	t127	t127 (1 month and 6 months)
C2	t127	t084 (2 days)
C5	t065	t065 (1 and 6 months)
C6	t002	t7568 (3 months), t002 (6 months)
C7	t3884	t3884 (1 and 3 months)

Table 1. *S. aureus spa*-types before and after decolonisation in 5 healthy carriers with *S. aureus* recolonisation.

Disruption and recovery of the nasal microbiota after decolonisation

Before decolonisation, nine dominant bacterial species in nasal microbiota, including *S. aureus*, *S. epidermidis*, *D. pigrum*, *Moraxella nonliquefaciens*, *C. acnes* and 4 *Corynebacteria* species were identified (Figure 3A, C; see details for each participant in Supplementary Figure 3). *D. pigrum*, a common taxon found in the anterior nares, was particularly abundant and prevalent in noncarriers. *C. propinquum* was present in both groups and was in average 15% more abundant in noncarriers. Mupirocin-sensitive species, including *S. aureus* and *S. epidermidis*, *D. pigrum* and *M. nonliquefaciens*, were virtually removed from the microbiota after decolonisation, while mupirocin-resistant corynebacteria and *C. acnes* remained substantially abundant^{28,29}. After decolonisation, the average proportion of *C. pseudodiphtheriticum* in noncarriers, but not carriers, increased 10-fold after 7 days and the proportion of *S. epidermidis* increased 10-fold after 1 month. At other time points, the average proportions of *C. pseudodiphtheriticum* and *S. epidermidis* were comparable in carriers and noncarriers. In the 2 carriers and 4 noncarriers colonised with more than 10% of *D. pigrum* (Supp. Figure 2), 1 was recolonised with *D. pigrum* after 1 month, 2 after 3 months and all after 6 months. *M. nonliquefaciens*, which was observed in 2 noncarriers, recolonised only 1 participant after 6 months. The median time to recolonisation with *D. pigrum* and *M. nonliquefaciens*, 2 major taxa present in noncarriers, was 6 months. In contrast, the median time to recolonisation of *S. aureus* in carriers was 3 months. Microbiota profiles of individual participants are shown in Supplementary figure 3.

To provide a more synthetic assessment of decolonisation-induced changes of the microbial community structure, we computed the Bray-Curtis dissimilarity of the species assemblage at each time point, relative to the initial D0 time point in the same patient (Figure 3B, D). The average Bray-Curtis dissimilarity was maximal immediately after decolonisation in both carriers and noncarriers, denoting the most perturbed state of the microbiota. Strikingly, the dissimilarity decreased sharply in carriers but remained mostly stable in noncarriers, indicating that the microbiota of carriers (partially) reverted toward their initial state faster than in noncarriers, in line with faster recolonisation by *S. aureus* compared to the dominant species found in noncarriers. After 6 months, the average dissimilarities from the initial state remained substantial (~0.5–0.7) both in carriers and noncarriers (Figure 3B, D). Importantly, the evolution of population structure varied strongly across participants (see dashed lines in Figure 3B, D), with microbiota recovery patterns ranging from fast recovery (dissimilarity <0.2 after 1 month) to virtually no recovery (dissimilarity >0.9 after 6 months) both in carriers and noncarriers.

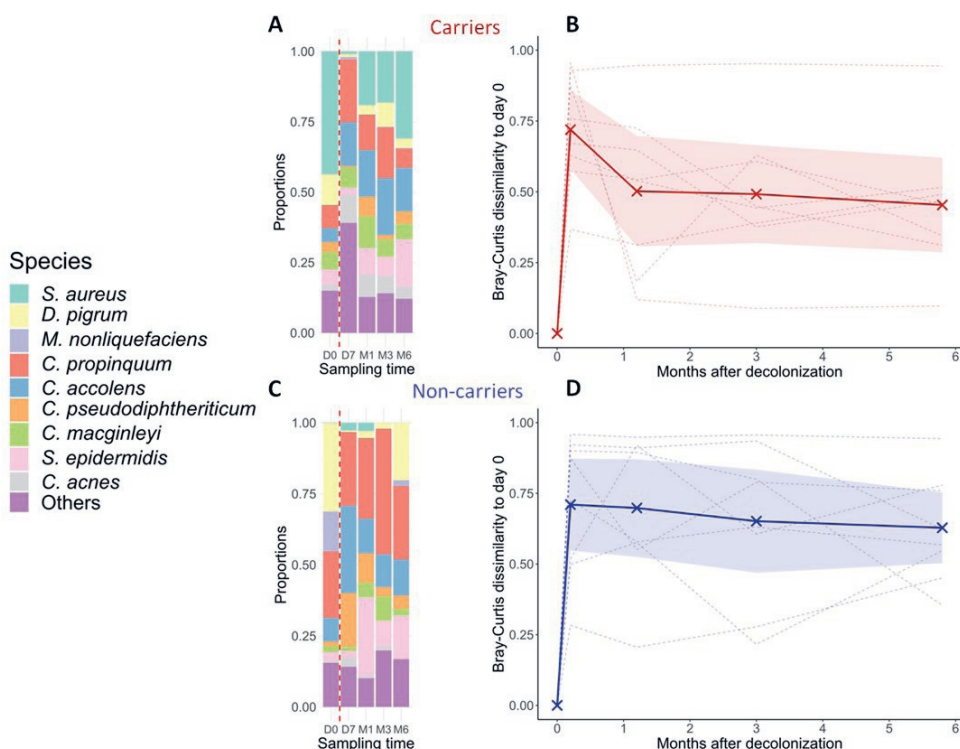


Figure 3. Evolution of the community structure of the nasal microbiota before and after mupirocin decolonisation in *S. aureus* carriers and noncarriers.

Shown are diversity bar plots of average species proportions (A, C) and the dissimilarity of these proportions (B, D) in each patient (dashed lines) and in average (solid lines; shaded area is the 95% confidence band of the mean). A high value for the Bray-Curtis dissimilarity indicates large difference in community structure relative to the initial state of the microbiota before decolonisation. Nasal samples were taken immediately before decolonisation (D0) and after 7 days (D7) and 1 (M1), 3 (M3), and 6 (M6) months in 8 *S. aureus* carriers (A, B) and noncarriers (C, D). Bacteria full names are, in order: *Staphylococcus aureus*, *Dolosigranulum pigrum*, *Moraxella nonliquefaciens*, *Corynebacterium propinquum*, *Corynebacterium accolens*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium macginleyi*, *Staphylococcus epidermidis*, *Cutibacterium acnes*.

Discussion

In this longitudinal study of *S. aureus* carriers and noncarriers undergoing nasal mupirocin decolonisation, we find that *S. aureus* recolonisation in carriers occurred more rapidly than recolonisation by the dominant species in noncarriers. These findings highlight the transient efficacy of the *S. aureus* decolonisation procedure and the strong adaptation of *S. aureus* to the nasal niche.

Next to one case of failed decolonisation, we observed frequent recolonisation with *S. aureus* during the 6-month follow-up period. The time to recolonisation ranged from 1 month to 3 months, in line with previous observations in which the delay to recolonisation ranged from 2 weeks to 6 months after mupirocin treatment³⁰. In another longitudinal study collecting samples of 571 participants

every 2 months for >2 years, anti-staphylococcal antibiotics increased the rate of *S. aureus* acquisition within 4 months after treatment ³¹, suggesting that microbiota disruption by antibiotics facilitates the invasion by *S. aureus*. In our study, 4 of 5 cases of recolonisation eventually occurred with the same *spa*-type as isolated from the carrier initially. However, transient colonisation with another *spa*-type was also demonstrated. This is in accordance with other studies showing longitudinal carriage of the same strain, with intermittent carriage of other strains as well ^{31–33}. While longitudinal studies suggest that loss and acquisition of *S. aureus* occur as natural events ^{31,33}, another reason for recolonisation could be the lack of successful decolonisation. Resistance to the decolonisation treatment could facilitate recolonisation. However, as Dutch national surveillance for resistance in *S. aureus* has shown low levels of mupirocin resistance (1%) ³⁴, it seems unlikely this would drive recolonisation in our study participants. Recolonisation from an untreated extra-nasal body site, such as the pharynx, or through household members is a more probable explanation.

Next to the loss of *S. aureus*, decolonisation caused the immediate removal of *S. epidermidis*, *D. pigrum* and *M. nonliquefaciens* from the nose. In noncarriers, a trend towards a higher abundance of *C. propinquum* was observed, while an increase in *C. accolens* and *S. epidermidis* was shown in effectively decolonised carriers. Indeed, mupirocin treatment has been previously tied to an increase of the relative abundance of (unclassified) corynebacteria and *C. acnes*, along with a decrease of *S. epidermidis* abundance ³⁵. Together, these results imply a rearrangement of the nasal microbiota after decolonisation treatment and the removal of mupirocin-susceptible species including *S. aureus*, allowing new taxa to invade the nasal niche.

Our study has limitations beyond its small sample size. To enhance study participation, we adopted a self-sampling strategy which allowed participants to send in samples through regular mail service. This method has been found appropriate for detection of *S. aureus* previously ^{36,37}. Nevertheless, delayed transport caused 20% of samples to be processed > 48 hours after sampling. As only 3 of 27 delayed samples in carriers were culture-negative, the risk of false negative *S. aureus* cultures due to transport can be considered low. However, the impact of delay on metabarcoding approaches is unknown. Nevertheless, delayed transport had no effect on the overall recolonisation results in this study.

Discrepancies were found between quantitative culture results and RNA metabarcoding regarding the presence of *S. aureus* in the post-decolonisation samples. We defined recolonisation based on a *S. aureus* positive culture (>8 CFU/mL) after decolonisation, consistent with our definition of *S. aureus* carriage. The varying nasal bacterial load, the intrinsic microbiota composition as well as potential influence of transport to the sequencing facility added to the multi-step RNA metabarcoding analyses are amongst the many factors explaining such differences with the culture results. Both methods agreed about recolonisation status in 3 carriers only.

Overall, our findings highlight the sensitivity of the nasal bacterial community to mupirocin treatment and stress the fact that the decolonisation target, namely *S. aureus*, re-enters the nasal niche comparably faster than the dominant species in noncarriers. This supports the current use of mupirocin as a short-term prevention procedure preceding an identified at-risk intervention, rather than a means of eliminating circulating *S. aureus* isolates.

Acknowledgements

We thank all participants for their contribution to this study. Special acknowledgements to Willem van Wamel, Patricia Vellekoop and Willemien Zandijk for laboratory assistance, as well as to Agnès Nguyen (Biofidal, Vaulx-en-Velin, France), for insightful discussions and assistance regarding the metabarcoding strategy used in this paper.

This work was part of the MACOTRA project, funded by ZonMw grant number 547001006 and ANR grant number 16-JPEC-0006. We acknowledge all members of the MACOTRA study group, consisting of: Valérie O. Baede ¹, Anaïs Barray ^{2,3}, Sake J. de Vlas ⁴, Anneke S. de Vos ⁴, Arya Gupta ⁵, Antoni P. A. Hendrickx ⁶, Gwenan M. Knight ⁷, Mirjam E. E. Kretzschmar ^{6,8}, Gérard Lina ^{2,3}, Jodi A. Lindsay ⁵, Jean-Philippe Rasigade ^{2,3}, Leo Schouls ⁶, Mehri Tavakol ¹, Margreet C. Vos ¹, Willem J. B. van Wamel ¹, Adam A. Witney ⁵

References

1. Wertheim, H. F. *et al.* The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* **5**, 751–762 (2005).
2. Sakr, A., Brégeon, F., Mège, J.-L., Rolain, J.-M. & Blin, O. *Staphylococcus aureus* Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Front. Microbiol.* **9**, 2419 (2018).
3. Bode, L. G. M. *et al.* Preventing Surgical-Site Infections in Nasal Carriers of *Staphylococcus aureus*. *N. Engl. J. Med.* **362**, 9–17 (2010).
4. World Health Organization. Evidence-Based Recommendations on Measures for the Prevention of Surgical Site Infection. in *Global Guidelines for the Prevention of Surgical Site Infection* (2018).
5. Kalenic, S. *et al.* Comparison of recommendations in national/regional Guidelines for prevention and control of MRSA in thirteen European countries. *Int. J. Infect. Control* **6**, 2 (2010).
6. Yan, M. *et al.* Nasal Microenvironments and Interspecific Interactions Influence Nasal Microbiota Complexity and *S. aureus* Carriage. *Cell Host Microbe* **14**, 631–640 (2013).
7. Liu, C. M. *et al.* *Staphylococcus aureus* and the ecology of the nasal microbiome. *Sci. Adv.* **1**, e1400216 (2015).
8. Krismer, B., Weidenmaier, C., Zipperer, A. & Peschel, A. The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nat. Rev. Microbiol.* **15**, 675–687 (2017).
9. Ramakrishnan, V. R. *et al.* Determinants of the Nasal Microbiome: Pilot Study of Effects of Intranasal Medication Use. *Allergy Rhinol.* **9**, 215265671878951 (2018).
10. Burnham, C.-A. D. *et al.* Topical Decolonization Does Not Eradicate the Skin Microbiota of Community-Dwelling or Hospitalized Adults. *Antimicrob. Agents Chemother.* **60**, 7303–7312 (2016).
11. Nouwen, J. L. *et al.* Predicting the *Staphylococcus aureus* Nasal Carrier State: Derivation and Validation of a ‘Culture Rule’. *Clin. Infect. Dis.* **39**, 806–811 (2004).
12. Aires-de-Sousa, M. *et al.* High Interlaboratory Reproducibility of DNA Sequence-Based Typing of Bacteria in a Multicenter Study. *J. Clin. Microbiol.* **44**, 619–621 (2006).
13. Li, R. *et al.* Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Sci. Rep.* **7**, 5752 (2017).
14. Kaplan, H. B., Miranda, J. A., Gogola, G. R., Gomez, K. & Ambrose, C. G. Persistence of bacterial DNA in orthopedic infections. *Diagn. Microbiol. Infect. Dis.* **91**, 136–140 (2018).
15. Levy-Booth, D. J. *et al.* Cycling of extracellular DNA in the soil environment. *Soil Biol. Biochem.* **39**, 2977–2991 (2007).
16. Fittipaldi, M., Nocker, A. & Codony, F. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *J. Microbiol. Methods* **91**, 276–289 (2012).
17. Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584 (2016).
18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
19. Camacho, C. *et al.* BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421 (2009).
20. R Core Team. R: A language and environment for statistical computing. (2017).

21. Wickham, H., François, R., Henry, L. & Müller, K. *dplyr: A Grammar of Data Manipulation*. (2021).
22. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (2016).
23. Oksanen, J. *et al. vegan: Community Ecology Package*. (2020).
24. Dhariwal, A. *et al.* MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **45**, W180–W188 (2017).
25. Chong, J., Liu, P., Zhou, G. & Xia, J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* **15**, 799–821 (2020).
26. Price, A., Sarween, N., Gupta, I. & Baharani, J. Meticillin-resistant *Staphylococcus aureus* and meticillin-susceptible *Staphylococcus aureus* screening in a cohort of haemodialysis patients: carriage, demographics and outcomes. *J. Hosp. Infect.* **90**, 22–27 (2015).
27. Pérez-Cobas, A. E. *et al.* Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut* **62**, 1591–1601 (2013).
28. Sum, S., Park, H.-M. & Oh, J. Y. High-level mupirocin resistance in Gram-positive bacteria isolated from diseased companion animals. *J. Vet. Sci.* **21**, (2020).
29. Sutcliffe, J. *et al.* Susceptibility of *Cutibacterium acnes* to topical minocycline foam. *Anaerobe* **62**, 102169 (2020).
30. Mody, L., Kauffman, C. A., McNeil, S. A., Galecki, A. T. & Bradley, S. F. Mupirocin-Based Decolonization of *Staphylococcus aureus* Carriers in Residents of 2 Long-Term Care Facilities: A Randomized, Double-Blind, Placebo-Controlled Trial. *Clin. Infect. Dis.* **37**, 1467–1474 (2003).
31. Miller, R. R. *et al.* Dynamics of acquisition and loss of carriage of *Staphylococcus aureus* strains in the community: The effect of clonal complex. *J. Infect.* **68**, 426–439 (2014).
32. Sakwinska, O. *et al.* Ecological Temporal Stability of *Staphylococcus aureus* Nasal Carriage. *J. Clin. Microbiol.* **48**, 2724–2728 (2010).
33. Muthukrishnan, G. *et al.* Longitudinal genetic analyses of *Staphylococcus aureus* nasal carriage dynamics in a diverse population. *BMC Infect. Dis.* **13**, 221 (2013).
34. Stichting Werkgroep Antibioticabeleid & Rijksinstituut voor Volksgezondheid en Milieu (RIVM), C. I. (CIb). *NETHMAP 2012. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands*. <https://swab.nl/nl/nethmap> (2012).
35. Roghmann, M.-C. *et al.* Effect of mupirocin for *Staphylococcus aureus* decolonization on the microbiome of the nose and throat in community and nursing home dwelling adults. *PLoS One* **16**, e0252004 (2021).
36. van Cleef, B. A., van Rijen, M., Ferket, M. & Kluytmans, J. A. Self-sampling is appropriate for detection of *Staphylococcus aureus*: a validation study. *Antimicrob. Resist. Infect. Control* **1**, 34 (2012).
37. Harrison, E. M. *et al.* Validation of self-administered nasal swabs and postage for the isolation of *Staphylococcus aureus*. *J. Med. Microbiol.* **65**, 1434–1437 (2016).

Supplementary material

Supplementary Table S1. Risk factors for *S. aureus* acquisition

Supplementary Figure 1. Rarefaction plot

Supplementary Figure 2. *S. aureus* culture dynamics in carriers and noncarriers

Supplementary Figure 3. Nasal microbiota of study participants in time

Chapter 7

Summarising discussion and future work

Goal of this thesis

S. aureus is an important human pathogen, characterised by its many virulence factors which enhance its pathogenic nature. Combined with the ability to circumvent antimicrobial therapy, through the acquisition of antimicrobial resistance genes, MRSA has evolved to one of the most important antimicrobial-resistant pathogens worldwide. Most of the MRSA burden is attributed to only a few genetic lineages, which dissemination was so successful that they can be considered pandemic clones. Nevertheless, not all MRSA clusters have been successful in transmission. Some clones, such as USA300, are very successful in certain parts of the world, but are unable to spread extensively in other geographic areas, despite frequent introduction¹. Even countries in close geographic proximity to each other can show considerable differences in MRSA epidemiology. To reduce the MRSA burden and to mitigate MRSA transmission, it is important to understand which factors contribute to MRSA transmission success. In this thesis, a wide range of potential factors is investigated, including bacterial, environmental and host factors. Additionally, the influence of public health policy and management is discussed. In the first part of this chapter, the main findings of this thesis will be discussed in context of relevant scientific literature, followed by the main conclusion. In the second part of this chapter, additional thoughts on other potential influential factors are shared, followed by important considerations for future work on MRSA transmission success.

Main findings of this thesis

In **Chapter 2**, the association between MRSA transmission success and resistance against a range of antimicrobial drugs was investigated. We established that fluoroquinolone, macrolide and mupirocin resistance was associated with MRSA success, while gentamicin, rifampicin and trimethoprim resistance was associated with sporadic MRSA. These findings confirm the earlier studies describing associations found for HA-MRSA with ciprofloxacin and macrolide resistance²⁻⁴. To investigate if antimicrobials associated with success were also used in higher frequencies, we studied the relationship between MRSA incidence and antibiotic usage across 29 European countries using ESAC-Net data. We found that usage of beta-lactams, fluoroquinolones and macrolides across European countries correlated with higher MRSA incidence and beta-lactamase sensitive penicillins correlated with lower MRSA incidence. Together, these results support the suggestion that **the selective pressure of antimicrobial usage can have a strong impact on MRSA incidence and contribute to the success of epidemic clones**, especially for fluoroquinolones and macrolides which showed an association with epidemic success in our study. This again underlines the importance of prudent use of antimicrobial drugs to reduce selection pressure and thereby reduce the acquisition and further spread of MRSA.

In **Chapter 2**, we attempted to establish a robust strain collection of epidemic and sporadic MRSA isolates originating from France, the Netherlands and the United Kingdom. However, we experienced that methodologies regarding MRSA surveillance and collection of MRSA isolates were very different between countries. Therefore, we had to adopt operational definitions of transmission success tailored to each country's specific situation. These operational definitions did not completely capture epidemic success as suggested by phylogenetic analysis, thereby highlighting the difficulty of identifying epidemic and sporadic MRSA isolates in clinical practice. As a result, selection bias and misclassification of isolates in our

MRSA collection could have influenced our ability to determine markers of epidemiological success. To improve identification of epidemic and sporadic MRSA, **we conclude that MRSA surveillance should be harmonised across countries and more comprehensive microbiological and epidemiological datasets should be composed to study MRSA transmission success.**

In order to evaluate the diversity of MRSA surveillance, a structured survey was conducted among 24 staphylococcal reference laboratories and MRSA surveillance programmes across the world. The outcomes of this survey are presented in **Chapter 3**. We found large differences in the structure of the surveillance programmes as well as the collection of epidemiological and/or microbiological data and samples between countries. The most important reasons for the large differences in surveillance were diverse surveillance goals, financial constraints and limited laboratory capacity in relation to MRSA prevalence. Often, the focus of surveillance was on the collection of clinical isolates, most importantly bloodstream infections (BSI). Only three countries performed active systemic surveillance of carriage isolates. This was surprising because severe invasive infections such as BSIs are often preceded by carriage^{5,6}, which means that carriage isolates can function as a sentinel for emerging MRSA clones. MRSA carriage in the community also contributes substantially to MRSA transmission networks⁷. To get a better view on emerging MRSA clones and MRSA transmission in the community, inclusion of carriage isolates in MRSA surveillance is required. As a first step, we recommend to include community-acquired MRSA such as isolates from skin and soft-tissue infections. Another concern regarding MRSA surveillance was the lack of centralised biobanks and integration of microbiological and epidemiological data. Systematic surveillance combined with consistent genotyping of all isolates can reveal the emergence of new MRSA clones, as shown for the introduction of USA300 in the United Kingdom⁸. Additionally, long-term monitoring enables the evaluation of new resistance profiles and the impact of nationwide or even international health policies on MRSA transmission, next to the possibility for extensive risk factor analysis for MRSA acquisition and infection when combined with meta-data reports. This would contribute to new guidelines for infection prevention. Other studies have suggested similar actions to improve surveillance^{9–11}. In conclusion, **we encountered large differences in MRSA surveillance between countries, ranging from minimal surveillance to extensive systems** in which all MRSA isolates were collected accompanied by extensive epidemiological datasets. In order to move towards harmonisation of MRSA surveillance, we proposed the following: (1) to include all bloodstream-infection isolates and a representative number of isolates from skin and soft-tissue infections in proportion to MRSA prevalence, (2) to collect and integrate microbiological and epidemiological data within national systems and (3) to implement central biobanks at the national level. Additionally, it is desirable to perform molecular characterisation, preferably whole-genome sequencing, of a well-defined sample of MRSA isolates in proportion to MRSA prevalence. To establish an international surveillance network with harmonised methodologies, clear guidelines and regular evaluation are needed.

MRSA is shed into the environment by infected patients or carriers. As a result, objects in the environment can become contaminated and form a new source of infection, a fomite¹². The ability of MRSA to survive on fomites in greater numbers would enhance transmission of MRSA to new hosts and could benefit the transmission success of epidemic strains. In **Chapter 4**, we describe the development of an *in-vitro* dehydration assay using isothermal microcalorimetry in combination with a new mathematical algorithm to study dehydration tolerance of epidemic versus

sporadic MRSA strains in a systematic and robust manner. Using this method, we found that exposure to dehydration resulted in similar survival numbers for epidemic and sporadic MRSA strains. After 7 days, viable numbers decreased by approximately $1 \log_{10}$ for both epidemic and sporadic MRSA strains. This implies that dehydration tolerance is an important trait for MRSA, and *S. aureus* in general, to survive in the environment. Indeed, *S. aureus* strain SH1000 has been shown to survive dehydration on polypropylene for more than 3 years at 25 °C in a laboratory setting¹³. Many bacterial species have adapted to tolerate dehydration, for example by entering a dormant state or producing extracellular polymeric substances (EPS) to form a biofilm¹⁴. Both protective strategies are employed by *S. aureus*. Our own experiments using isothermal microcalorimetry demonstrated how MRSA enter a state of metabolic dormancy during dehydration, where heat flow decreased below the detection limit (data not shown). Also, the formation of dry surface biofilms has been demonstrated for a wide range of hospital surfaces^{15–17}. Swabbing or rinsing the area of interest may not be sufficient to yield positive culture results and prove the presence of dry surface biofilms¹⁷. Nevertheless, once established, they can be very difficult to remove. Chlorine treatment up to 20.000 ppm reduced plate counts by $7 \log_{10}$ bacteria, but this was not enough to prevent biofilm recovery and subsequent release of planktonic *S. aureus*¹⁸. Similar results were obtained using heat treatment such as autoclaving at 121 °C for 30 min¹⁹. As presented in **Chapter 4**, we found an inoculum effect on bacterial survival, with higher survival numbers in higher inocula. We could not confirm differences between genetic lineages due to lack of statistical power. Taken together, **we conclude that dehydration tolerance is a universal trait of *S. aureus* and thus MRSA, which is subjected to an inoculum effect. Its influence on the difference in transmission success of epidemic versus sporadic MRSA is absent or small.** Nevertheless, we did not test if epidemic and sporadic MRSA differ in their ability to recover from dehydration and subsequently infect a new patient after dehydration. Overall, it remains important to prevent environmental contamination and apply adequate cleaning and disinfection measures in the case of a (highly) contaminated spill.

Besides acquisition from environmental sources, MRSA transmission between people is facilitated by carriage, either in the nose or on skin^{20,21}. Therefore, the ability of MRSA to survive on skin plays an important role in MRSA transmission. In **Chapter 5**, the survival of epidemic and sporadic MRSA on human skin was investigated. The host-pathogen interaction was further evaluated through the expression of several antimicrobial peptides, cytokines and chemokines by keratinocytes and the production of toxins, immune evasion proteins and other virulence factors by MRSA. For epidermal models based on a human keratinocyte cell line, no differences in survival were found between epidemic and sporadic strains. However, on epidermal models derived from primary human keratinocytes of different donors, lower survival of sporadic strains was found. This finding indicates that survival of MRSA on skin is influenced by host variation. This is consistent with various artificial *S. aureus* inoculation studies, which indicated that *S. aureus* carriage was the result of an optimal fit between colonising strain and host^{22,23}. While gene polymorphisms do not completely explain persistent carriage²⁴, host genetics can have a strong influence on an individual's susceptibility to infectious diseases, for example through variation in the major histocompatibility complex (MHC)²⁵. Indeed, a genome-wide association study (GWAS) among >50.000 *S. aureus* cases and matched controls identified polymorphisms in HLA class II genes that were associated with *S. aureus* infection²⁶. This supports the possibility

that some human hosts are more susceptible to *S. aureus* and MRSA infection than others and thus potentially influence transmission.

Chapter 5 also describes how expression of pore-forming toxins Hla, LukED, PVL (LukS) and HlgB upon interaction with keratinocytes of a human cell line was mostly restricted to epidemic strains. This was also the case for extracellular adherence protein Eap. Alpha-toxin (Hla), or alpha-hemolysin, is a pore-forming toxin which causes lysis of the host cell upon binding to the ADAM10 receptor ²⁷. This receptor is expressed on various immune cells, such as monocytes, macrophages and lymphocytes, but also epithelial cells including keratinocytes ^{27,28}. Most *S. aureus* strains harbour the *hla* gene ²⁹, but its expression is dependent on multiple regulatory systems, including the accessory gene regulator (*agr*), Sar family and SaeR/S two-component system ²⁷. Hla expression was shown to be higher in community-associated MRSA strains compared to hospital-associated MRSA strains ³⁰ and the enhanced virulence of epidemic MRSA USA300 strain compared to a USA400 strain in a rat pneumonia model was associated with increased expression of Hla ³¹. HlgB is the F-subunit of gamma-hemolysin, which is either combined with S-subunit HlgA to form the classical gamma-hemolysin HlgAB or S-subunit HlgC to form HlgCB ^{27,28,32}. Both have cytolytic activity towards leukocytes, i.e., monocytes and neutrophils, but HlgAB is particularly toxic for erythrocytes as well ^{27,28}. Most colonisation strains carry the *hlgACB* locus, but there is no known association with certain infections ²⁷. LukED is another leukotoxin that is cytotoxic against monocytes, neutrophils, dendritic cells and also T-lymphocytes ²⁷. This toxin is highly prevalent in hospital- as well as community-acquired isolates ²⁷. PVL (LukS) is cytotoxic against monocytes and neutrophils and has been strongly associated with community-acquired MRSA isolates ^{33,34}. Extracellular adherence protein (Eap) plays an important role in *S. aureus* immune evasion. First, Eap is an inhibitor of the classical and lectin complement pathways through the inhibition of C3 convertase ³⁵. Furthermore, it reduces the formation of neutrophil extracellular traps and both Eap and its homologs EapH1 and EapH2 inhibit neutrophil serine proteases, thus promoting *S. aureus* survival during infection ^{36,37}. Also, the interaction between *S. aureus* and keratinocytes is affected by secreted Eap. Authentic human wounds exhibit high levels of *eap* transcription, suggesting interference with wound healing ³⁸. Primary keratinocytes and cell cultures of the HaCat cell line both showed increased bacterial adhesion and internalisation after treatment with Eap ³⁹. Furthermore, *in vitro* experiments with HaCat cell cultures show that Eap has the ability to alter the proliferation, cell morphology and migration of human keratinocytes ⁴⁰. Eap is present in high proportions (>90%) in both carriage as well as invasive *S. aureus* isolates ⁴¹. In our study, the expression of Hla, HlgB, LukED, PVL (LukS) and Eap did not lead to a higher survival of epidemic strains. However, it is likely that expression of these proteins will benefit the strain's survival upon interaction with the host's immune cells. For Hla, LukED and PVL, higher expression is found in CA-MRSA and/or HA-MRSA strains, possibly more frequently in epidemic strains ^{27,30,31,33,34}. This suggests that these bacterial factors might play a role in the transmission success of MRSA. Furthermore, we observed passage of the bacteria through the cell culture filter, which happened more quickly in epidemic strains of CC5 and CC8. This passage was mediated through the interaction between bacteria and keratinocytes, indicating that it was likely facilitated by factors produced either by keratinocytes and/or the bacteria upon this interaction. Unfortunately, in our study, we were unable to show any association of filter passage with production of either immune or bacterial virulence factors. To summarise, **we conclude that both bacterial and host factors influence the survival of MRSA on skin.**

This highlights the complexity of MRSA transmission success and indicates that reasons for transmission success are not solely dependent on the strain, but it is likely that the unique interaction between host and pathogen is most important.

Host factors were further investigated in **Chapter 6**. Here, the longitudinal influence of *S. aureus*-targeted decolonisation treatment on nasal microbial communities in *S. aureus* carriers and noncarriers was evaluated. Additionally, we investigated the possibility of *S. aureus* recolonisation in these individuals. We found that decolonisation treatment was successful in 7 out of 8 carriers. Nevertheless, recolonisation occurred in 5 individuals within 1-3 months, which was consistent with other literature ⁴². In the majority of cases, the same *spa*-type was found during recolonisation as before decolonisation, possibly indicating that the carrier was recolonised with his own strain. Transient colonisation with different *spa*-types occurred in a few carriers as well, as was observed in various longitudinal studies of *S. aureus* carriage ⁴³⁻⁴⁵. Noncarriers also picked up *S. aureus* occasionally, but this did not result in carriage in our study. Decolonisation treatment had a strong effect on the composition of nasal microbial communities, in carriers as well as noncarriers. *S. aureus* and *Staphylococcus epidermidis* were removed, as well as *Dolosigranulum pigrum* and *Moraxella nonliquefaciens*. The latter were the dominant species in the noncarrier microbiota. Shortly after decolonisation, the nasal microbiota was dominated by mupirocin-resistant corynebacteria in both carriers and noncarriers. Recovery of the nasal microbiota was slow. Bray-Curtis dissimilarity was still substantial after 6 months in both groups. The median recolonisation time of the dominant species was also much longer in noncarriers than carriers, indicating the vast effect of decolonisation treatment on the nasal microbiota next to the resilience of *S. aureus*. From this study, **we conclude that *S. aureus*-targeted decolonisation treatment has a long-lasting effect on the composition of the nasal microbiota in *S. aureus* carriers as well as noncarriers.** For the gut microbiota, it has been shown that loss of biodiversity due to antimicrobial treatment can increase the susceptibility to pathogenic infections ⁴⁶. Another study showed increased rates of *S. aureus* acquisition within 4 months after treatment with anti-staphylococcal antibiotics ⁴⁵. Also in our study, we found positive *S. aureus* cultures after decolonisation treatment showing how its opportunistic behaviour enables *S. aureus* to benefit from microbiota disruption. It is therefore important for clinicians to acknowledge that ***S. aureus* - targeted treatment will have a long-lasting effect on noncarriers as well and should only be used in carriers based on medical indication and not at all in noncarriers.**

Based on the findings in this thesis, we established that multiple bacterial and host factors influence transmission of certain (epidemic) clones. Additionally, healthcare policies affect transmission as well. **We conclude that MRSA transmission success is multifactorial and depends on a large array of intrinsic and external factors.**

Final thoughts and recommendations for future work

During the work for this thesis, several other issues came to mind that could influence epidemiological success of MRSA directly or indirectly. These issues are discussed below, followed by recommendations for future work.

First, it is important to note that epidemiological success could be the result of a combination of factors with a synergistic effect. Most studies investigating the success of epidemic clones have limited their analyses to individual (genomic) factors, while there are examples of co-occurring genes that enhance each other's function, such as the immune evasion cluster. This cluster encodes for chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN) and staphylokinase (SAK)⁴⁷, which complement each other's role in immune evasion^{48–50}. Also, carriage of multiple resistance genes on mobile genetic elements can lead to a synergistic effect through co-resistance⁵¹. In a similar fashion, other gene combinations could lead to epidemiological success. Therefore, studies should not limit their analyses to individual gene associations, but also consider combinations of synergistic genes, for example through gene network analysis. Here, it is also important to acknowledge that success factors likely vary for different clones, some may contribute only a little in the successful transmission of certain clones, while can be vital for the success of others. To identify potential synergistic gene clusters that could differ between MRSA clones, a large genomic dataset is needed for the clones of interest.

Even when clones carry the same genes, they can still show different responses upon interaction with the host or environment, due to differences in gene regulation. Gene expression in *S. aureus* is regulated by many regulatory systems, such as the accessory gene regulator (*Agr*) and the staphylococcal accessory regulator (*sar*)⁵². These systems are highly complex and subjected to many environmental and metabolic cues that can trigger up- or downregulation of genes^{52–55}. Plenty of such cues are present in the host during colonisation or infection^{54,55}, meaning that gene expression during colonisation or infection can vary across MRSA clones depending on gene regulation. Variations in gene expression could lead to different behaviour during colonisation or infection and ultimately influence transmission and epidemiological success. Unfortunately, it can be difficult to mimic the complex interaction between bacteria and host or environment with the relevant regulatory cues and subsequent response in a laboratory setting, making it difficult to study the natural behaviour of MRSA clones as present *in vivo*. To investigate the natural behaviour of MRSA clones during colonisation and infection that is responsible for epidemiological success, experimental models should mimic *in vivo* conditions as much as possible, to account for available external cues that will influence gene regulation and expression.

As discussed above, factors related to the human host are also important for sustained colonisation or infection and subsequent transmission. Perhaps most important is the immune response upon host-pathogen interaction. The immune system can vary between individuals due to host genetics as mentioned earlier, but is also affected by an individual's lifestyle through nutrition and acute or chronic stress among other factors^{56–58}. Immunosuppression and morbidities are risk factors for *S. aureus* colonisation and infection^{20,59} and high levels of these characteristics in a population can benefit successful transmission of an MRSA clone. These factors also contributed to the epidemiological success of health-care-associated MRSA clones. Therefore, to study the success of epidemic MRSA

clones, it is important to include the contribution of the immune status of patients or carriers which are affected by specific clones and the influence of optimised as well as impaired immune responses in experimental and theoretical infection models.

Besides the biological factors as described above, stochasticity, i.e., chance is an important factor in infectious disease dynamics ⁶⁰. Stochasticity will be more important for situations with fewer interactions ⁶⁰, for example for areas with lower population density or when less abundant or emerging clones are involved. Once a certain threshold for sustained transmission of that clone is established, chance will become less important and the deterministic characteristics of transmission will become more apparent.

To continue our search for factors that contribute to MRSA transmission success while accounting for the issues described above, improved data collection is needed. This includes epidemiological data collection through the compilation of a balanced and well-documented MRSA strain collection including relevant metadata such as host characteristics, as well as microbiological data collection through the design of experimental models that mimic *in vivo* MRSA colonisation and infection.

Recommendations regarding epidemiological data

To compile a balanced strain collection for the study of MRSA transmission factors, we should take three different epidemiological levels into account where differences in success markers might be found. Obviously, we should compare epidemic and sporadic strains overall. Additionally, sufficient data should be gathered for the clones of interest, because it is possible that success markers vary across clones. Furthermore, sufficient data should be gathered on other strains or clones within the same genetic lineage as the clone of interest, to correct for lineage-associated factors that could mask success markers of an epidemic clone in that lineage.

To identify epidemic and sporadic MRSA strains, clear definitions are needed. The definitions formulated by the MACOTRA consortium are listed in Box 1. Unfortunately, we were unable to use these definitions in this thesis due to the problems described in **Chapter 2**. Nevertheless, they can form a starting point for other researchers studying MRSA transmission success.

Furthermore, the quality of epidemiological metadata should be improved. For this, it is important to harmonise MRSA surveillance following the suggestions as discussed in **Chapter 3**. A standardised epidemiological metadata report should be completed, including information about the carrier or patient from whom the strain was isolated. To account for the influence of the patient's immune system on MRSA transmission success, immune status should be included on the report as well. Additionally, sufficient genomic data is needed for the identification of potential synergistic gene clusters.

Box 1Epidemic MRSA:

MRSA clone (defined by ST, MT or *spa*-type along with SCCmec type) involved in ≥ 2 independent outbreaks with transmission to ≥ 5 colonised or infected secondary cases (patients or health care workers) in the period 1990-present, either in health care institutes of ≥ 2 regions within the same country or repeatedly in the same health care institute.

Sporadic MRSA:

MRSA clone (defined by ST, MT or *spa*-type along with SCCmec type) not involved in an identified outbreak in the period 1990-present, despite longstanding exposure demonstrated by either:

1. the repeated isolation from one colonised or infected hospitalised patient or health care worker in the absence of MRSA outbreak preventive measures, and/or
2. the repeated isolation in unrelated infected patients over a period ≥ 4 years, and/or
3. the isolation as an accidental, genotypically unrelated finding in contact tracing in the context of an MRSA outbreak with "search and destroy" strategy or
4. epidemic behaviour in another country or another time period, i.e., before 1990.

Recommendations regarding microbiological data and experimental work

Obviously, a lot of work is needed to create experimental models that mimic *in vivo* colonisation or infection and account for variation in gene regulation as well as variation regarding host immunity. One should think wisely about local environmental and metabolic conditions in experimental models and work towards models that are true to the natural situation, accounting for the right type of human tissue, infection dose, oxygen availability, but also nutrient availability through culture media. To this end, it would be interesting to further investigate dehydration tolerance (as described in **Chapter 4**) and subsequent recovery in a more representative *in vivo* medium, such as a nasal medium (for MRSA transmission through sneezing or nose-picking) or plasma (for hospital spills).

To account for host variation, it is important to use models with primary cells from different donors or multiple cell lines. The epidermal models as described in **Chapter 5** could be modified to include the dermal layer and study the interaction with local immune cells.

Most of all, it is important to realise that ideal laboratory conditions with ideal bacterial growth will not represent the *in vivo* situation and will lack information about how MRSA acts under stressful conditions. Furthermore, small differences can lead to completely different results as illustrated by the inoculum effect in **Chapter 4**. The use of mathematical models could help to determine which conditions or variables are most important in the interaction between *S. aureus* and the host during colonisation or infection.

Overall, it is clear that MRSA transmission success is the result of the complex interplay between bacterium, host, host behaviour and the environment. This is a dynamic process and the versatility of *S. aureus* as a pathogen and its excellent abilities to survive under hostile conditions in the environment as well as in the host, lead to a highly varied and complex transmission process. I advocate to strive towards a more holistic approach to obtain a better understanding of the complex dynamics of MRSA transmission, with well-documented MRSA strain collections and experimental and theoretical models of colonisation and infection that mimic realistic *in vivo* situations.

Chapter 8

English summary

English summary

Staphylococcus aureus (*S. aureus*) is a bacterium residing on human skin and mucous membranes. As an opportunistic pathogen, it can cause a wide variety of infections, from superficial skin infections to invasive infections such as pneumonia, bone infections and sepsis. In general, these infections can be treated using antibiotics, but resistance against antibiotics has been rising. Of all known antimicrobial resistant pathogens, methicillin-resistant *S. aureus* (MRSA) currently causes the highest number of deaths worldwide ¹.

MRSA acquired resistance against antibiotics of the beta-lactam class through the uptake of the *mecA* gene. Only a limited number of genetic lineages of *S. aureus* took up this gene. The resulting MRSA clones spread to a pandemic level and are still dominant today. However, not all MRSA clones were able to spread so successfully. Next to that, some clones, such as the American USA300 clone, are very successful in their area of origin, but are still rare in other parts of the world despite frequent introduction. Other dominant clones are replaced by new emerging clones, such as happened in the UK with the replacement of EMRSA-16 by EMRSA-15.

It is still unclear which factors determine whether transmission of an MRSA clone will be successful. Bacterial factors, such as antimicrobial resistance genes or genes that lead to more virulent strains, could contribute to transmission success. Also, factors related to the human host could be important, such as an impaired immune system or competing microbes in the human microbiome. Another possibility is impaired survival of the bacteria in the environment, reducing the chance of subsequent transmission. External factors could be important, such as the policy concerning antibiotic usage and infection prevention.

To get a better understanding of the dynamic nature of MRSA epidemiology, more knowledge on the factors contributing to MRSA transmission success is needed. Ultimately, this would help to prevent MRSA transmission and subsequently reduce the number of MRSA infections.

This thesis focuses on the factors that are associated with the successful dissemination of epidemic MRSA clones compared to the limited dissemination of their sporadic counterparts. For this work, we used MRSA strains isolated from patients or carriers from France, the Netherlands and the United Kingdom, three countries with distinct MRSA epidemiology despite similar geographical and socio-economical characteristics.

In **Chapter 2** we investigated the relation between MRSA transmission success and resistance against a range of antimicrobial drugs. We found that epidemic MRSA were associated with resistance against fluoroquinolones, macrolides and mupirocin, while sporadic MRSA were associated with resistance against gentamicin, rifampicin and trimethoprim. Usage of penicillins, fluoroquinolones and macrolides across 29 European countries correlated with MRSA incidence. From these findings, we conclude that antimicrobial resistance and the selective pressure of antimicrobial usage contribute to epidemiological success of certain MRSA clones.

For the compilation of the MRSA strain collection investigated in **Chapter 2**, we had to identify epidemic and sporadic MRSA strains from France, the Netherlands and the United Kingdom based on different definitions. This was due to large differences in MRSA surveillance between these countries. Unfortunately, this

approach led to a selection bias in our MRSA collection and might have influenced our ability to determine markers of epidemiological success. To improve classification of epidemic and sporadic MRSA, we conclude that MRSA surveillance should be harmonised across countries.

In **Chapter 3**, we studied the diversity of MRSA surveillance programmes around the world. Large differences were found, both in the structure of the surveillance programmes as well as the type of data and samples that were collected. To improve MRSA surveillance on a global level, we conclude that surveillance should not only focus on bloodstream infections, but also include MRSA from skin and soft tissue infections. Also, defined microbiological data and epidemiological data should be collected and stored together at the national level and be accompanied by a central biobank of MRSA isolates. Furthermore, clear guidelines and regular evaluations of surveillance programmes are needed to move forward.

A potential factor influencing transmission success of MRSA is the ability to survive on contaminated surfaces and objects. In **Chapter 4**, we studied how well epidemic MRSA survived after 7 days exposure to dehydration compared to sporadic MRSA. We found similar survival numbers for both groups of MRSA. This implied that tolerance to dehydration is a universal trait of MRSA. We conclude that in our experimental setting, dehydration tolerance plays no or a minor role in the epidemiological success of MRSA clones.

Next, we focused on the ability of epidemic and sporadic MRSA to survive on human skin. In **Chapter 5**, we describe that MRSA survival was comparable on skin models constructed of an epidermal cell line, but epidemic MRSA survived better on some skin models based on skin from human donors. This implied an important role for host variation in the survival of MRSA on human skin. Both groups of MRSA strains induced a comparable immune response, but most bacterial toxins were exclusively produced by epidemic strains of MRSA. This indicated that epidemic MRSA exhibited more aggressive behaviour upon interaction with the skin than sporadic MRSA, potentially contributing to the transmission success of epidemic MRSA. We conclude that both host as well as bacterial factors influence the survival of MRSA on human skin.

In **Chapter 6**, we studied the longitudinal influence of *S. aureus*-targeted treatment on nasal carriage of *S. aureus* as well as the nasal microbiome composition. Treatment was successful in most *S. aureus* carriers, but *S. aureus*-positive samples were found within 1-3 months. Treatment had a strong effect on the composition of nasal microbial communities, in carriers as well as noncarriers. Recovery of the nasal microbiota was slow and took much more time in noncarriers than carriers. We conclude that *S. aureus*-targeted treatment has a long-lasting effect on the composition of the nasal microbiota in *S. aureus* carriers as well as noncarriers and should only be used in carriers based on medical indication and not at all in noncarriers.

To summarise, we established that multiple bacterial and host factors influence MRSA transmission and benefit certain (epidemic) clones. Additionally, antibiotic usage affects transmission as well. We conclude that MRSA transmission success is multifactorial and depends on a large array of intrinsic and external factors.

The factors that are actually important for the successful transmission of MRSA clones could be different for each clone. Some factors may contribute only a little in the successful transmission of certain clones, while they are vital for the

success of others. This means that transmission success is complex and difficult to decipher completely.

For future research, it is important that MRSA surveillance is improved and harmonised on a global scale. Additionally, laboratory experiments should be designed with a holistic approach in mind, to mimic realistic *in vivo* colonisation or infection and obtain a better understanding of the complex dynamics of MRSA transmission.

1. Murray, C. J. et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 (2022).

Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

Staphylococcus aureus is een bacterie die mensen vaak bij zich dragen op de huid en slijmvliezen. *S. aureus* is een opportunist en deze kan veel verschillende infecties veroorzaken, van lokale huidinfecties tot invasieve infecties, zoals longontsteking, botinfecties en sepsis. Zulke infecties worden behandeld met antibiotica, maar steeds vaker blijkt *S. aureus* resistent tegen deze behandelingen. Meticilline-resistente *S. aureus* (MRSA) veroorzaakt momenteel van alle bekende antibioticaresistente ziekteverwekkers het hoogste aantal sterfgevallen wereldwijd ¹.

MRSA verwierf resistentie tegen de meeste antibiotica van de beta-lactam klasse door de opname van het *mecA* gen. Onderzoek naar de herkomst van dit gen toonde aan dat het slechts door enkele genetische lijnen van *S. aureus* is opgenomen. Deze genetische lijnen, ook wel klonen genoemd, hebben zich wereldwijd verspreid en zijn ook nu nog verantwoordelijk voor het overgrote deel van infecties bij MRSA-patiënten. Echter, niet alle MRSA-klonen zijn in staat om zich op zulke grote schaal te verspreiden. Daarnaast zijn sommige klonen, zoals de Amerikaanse USA300 kloon, erg succesvol in hun oorspronkelijke herkomstgebied, maar ondanks herhaaldelijke introductie nog steeds zeldzaam in andere delen van de wereld. Er zijn ook voorbeelden van dominante klonen die worden verdrongen door een nieuwe opkomende kloon, zoals recent gebeurde in het Verenigd Koninkrijk waar EMRSA-16 verdrongen werd door EMRSA-15.

Het is nog onduidelijk welke factoren bepalend zijn voor de al dan niet succesvolle verspreiding van een MRSA-kloon. Bacteriële factoren kunnen hieraan bijdragen, zoals genen die leiden tot antibioticaresistentie of genen die MRSA virulenter maken. Maar ook factoren die betrekking hebben op de gastheer, de mens in dit geval, kunnen belangrijk zijn, zoals een verzwakt immuunsysteem of wedijverende microben in het menselijke microbiom. Ook het vermogen van MRSA om te overleven in de omgeving kan de kans op overdracht beïnvloeden. Daarnaast spelen externe factoren, zoals het beleid ten aanzien van antibioticagebruik en infectiepreventie, mogelijk ook een rol.

Om de epidemiologie van MRSA beter te begrijpen, is meer kennis nodig over alle factoren die bijdragen aan de succesvolle verspreiding van MRSA-klonen. Met deze kennis kunnen we hopelijk de verspreiding van MRSA in de toekomst vaker voorkomen en het aantal infecties met MRSA verminderen. In dit proefschrift staat het onderzoek naar factoren die ervoor zorgen dat sommige MRSA-klonen (epidemische klonen) zich meer verspreiden dan hun zeldzame tegenhangers centraal. Hierbij wordt gebruik gemaakt van MRSA-isolaten uit gezonde MRSA-dragers en MRSA-patiënten afkomstig uit drie landen waar de epidemiologie van MRSA uiteenloopt ondanks vergelijkbare geografische en sociaal-maatschappelijke omstandigheden, namelijk Frankrijk, Nederland en het Verenigd Koninkrijk.

In **Hoofdstuk 2** onderzochten we de relatie tussen succesvolle verspreiding van MRSA en resistentie tegen verschillende antibiotica. We stelden vast dat er een verband is tussen epidemische MRSA-klonen en resistentie tegen fluoroquinolonen, macroliden en mupirocine. Daarnaast vonden we een verband tussen zeldzame klonen van MRSA en resistentie tegen gentamicine, rifampicine en trimethoprim. Het gebruik van penicillinen, fluoroquinolonen en macroliden liet een correlatie zien met MRSA-incidentie in 29 Europese landen. Uit deze gegevens concluderen wij dat antibioticaresistentie en selectiedruk door antibioticagebruik bijdragen aan de succesvolle verspreiding van MRSA-klonen.

In **Hoofdstuk 2** werd een collectie van MRSA-isolaten uit Frankrijk, Nederland en het Verenigd Koninkrijk samengesteld. Door de grote verschillen in MRSA-surveillance tussen deze landen, moesten voor elk land verschillende definities gebruikt worden om epidemische en zeldzame MRSA-klonen te identificeren. Dit leidde tot een selectiebias in onze collectie, die mogelijk ons vermogen om kenmerken van succesvolle MRSA-overdracht waar te nemen heeft beïnvloed. We concluderen dat MRSA-surveillance in verschillende landen meer op elkaar afgestemd moet worden, om identificatie van epidemische en zeldzame MRSA-klonen te verbeteren.

In **Hoofdstuk 3** onderzochten we de diversiteit van MRSA-surveillance op wereldwijde schaal. We ontdekten grote verschillen, zowel in de opbouw van de surveillanceprogramma's als in de data en biologische monsters die werden verzameld. We concluderen dat MRSA-surveillance verbeterd kan worden door niet alleen MRSA-isolaten van bloedbaaninfecties te includeren, maar ook isolaten van huidinfecties. Daarnaast zouden microbiologische en epidemiologische data samen verzameld en bewaard moeten worden. Ook zou in elk land een centrale biobank voor MRSA-isolaten opgezet moeten worden. Om goede vooruitgang te boeken, zijn ook duidelijke richtlijnen en regelmatige evaluaties van alle surveillanceprogramma's nodig.

Het vermogen om te overleven op besmette oppervlakten en objecten draagt mogelijk bij aan een succesvolle overdracht van MRSA. In **Hoofdstuk 4** hebben we onderzocht of dit vermogen verschilde in epidemische en zeldzame MRSA-klonen, door beide groepen bloot te stellen aan uitdroging. We ontdekten geen verschillen tussen beide groepen, wat betekent dat tolerantie voor uitdroging belangrijk is voor MRSA in het algemeen. We concluderen dat in onze experimentele studie-opzet, tolerantie voor uitdroging geen of slechts een beperkte rol speelde in het verschil tussen MRSA-klonen om zich al dan niet succesvol te verspreiden.

In het volgende deel van dit proefschrift, **Hoofdstuk 5**, bestudeerden we het vermogen van MRSA om te overleven op de huid. We ontdekten dat epidemische en zeldzame MRSA-klonen even goed overleefden op huidmodellen van één cellijn, terwijl epidemische klonen beter overleefden op sommige huidmodellen gebaseerd op cellen van huiddonoren. Dit betekent dat de humane factor belangrijk is voor de overleving van MRSA op huid. We ontdekten een vergelijkbare immuunreactie van de huid tegen epidemische en zeldzame klonen. Daarentegen werden bacteriële toxinen bijna alleen geproduceerd door epidemische klonen. Dit betekent dat epidemische klonen zich in onze studie agressiever gedroegen in contact met de huid dan zeldzame klonen. Dit zou kunnen bijdragen aan het ontstaan van infecties en dus ook de overdracht van MRSA. We concluderen dat zowel humane als bacteriële factoren effect hebben op het vermogen van MRSA om te overleven op de huid.

In **Hoofdstuk 6** bekeken we het langdurig effect van een behandeling tegen *S. aureus* op *S. aureus*-dragerschap en de samenstelling van het neusmicrobioom. De behandeling was succesvol bij de meeste dragers van *S. aureus*, maar positieve neuskwelen werden wel weer binnen 1-3 maanden na behandeling gevonden. Verder had de behandeling een groot effect op de samenstelling van het neusmicrobioom in zowel dragers als niet-dragers. Het neusmicrobioom herstelde zich langzaam in dragers, maar nog langzamer in niet-dragers. We concluderen dat de behandeling tegen *S. aureus* een langdurig effect heeft op de samenstelling van

het neusmicrobioom in *S. aureus* dragers en niet-dragers. De behandeling tegen dragerschap van *S. aureus* met mupirocine zou alleen gebruikt moeten worden op medische indicatie in *S. aureus* dragers en helemaal niet in niet-dragers.

Samenvattend concluderen we dat meerdere bacteriële en gastheerfactoren en het gebruik van antibiotica van invloed zijn op MRSA-overdracht en mogelijke overdracht van epidemische klonen kunnen stimuleren. Succesvolle overdracht van MRSA is dus multifactorieel en afhankelijk van een groot scala aan intrinsieke en externe factoren.

Welke factoren daadwerkelijk van belang zijn voor succesvolle verspreiding van MRSA-klonen kan voor elke kloon anders zijn. Mogelijk zijn sommige factoren slechts beperkt van invloed op de overdracht van bepaalde klonen, maar cruciaal voor de overdracht van anderen. Het succes van MRSA-overdracht is dus een complex en moeilijk te ontcijferen fenomeen.

Om epidemische en zeldzame MRSA-klonen beter te identificeren, zou toekomstig onderzoek zich moeten richten op het verbeteren en harmoniseren van MRSA-surveillance in landen wereldwijd. Om een beter begrip te krijgen van de interactie tussen MRSA en zijn humane gastheer, is het belangrijk om kolonisatie- en infectiemodellen op te zetten vanuit een holistische benadering, waarbij de situatie *in vivo* zo realistisch mogelijk worden nagebootst.

1. Murray, C. J. *et al.* Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 (2022).

Appendices

Dankwoord

Curriculum vitae

List of publications

PhD portfolio

Dankwoord

Eindelijk is het dan zover, mijn proefschrift mag bijna naar de drukker. Maar na al het harde werk, is het eerst nog tijd om even stil te staan bij wat is geweest, om te bedenken hoever ik ben gekomen, om de successen te vieren en om iedereen te bedanken die hieraan heeft bijgedragen.

Allereerst Prof.dr. Vos. Beste Greet, jij bent een wervelwind aan enthousiasme en daadkracht. Jouw gastvrijheid zette de toon tijdens onze eerste MACOTRA study group meeting en heeft nog tot veel gezellige bijeenkomsten geleid in Rotterdam, Bilthoven, Londen, Lyon en later ook online. Tijdens onze overleggen kon ik vooral jouw filosofische insteek altijd erg waarderen. We hebben je door de pandemie helaas een tijd moeten missen, maar ik hoop dat je toch trots bent op het eindresultaat. Bedankt voor de waardevolle begeleiding in de afgelopen jaren.

Beste dr. van Wamel, beste Willem. Zo, het is toch maar weer gelukt! Bedankt dat je de afgelopen jaren de moeite hebt genomen om me op de kleine en grote successen te wijzen. Ik heb dan misschien niet helemaal hetzelfde soort hoofd, maar ik denk dat we tegenwoordig redelijk op één lijn zitten. We kunnen in ieder geval heel goed ouwehoeren samen, niet alleen over die rotbacteriën, maar ook over allerlei obscure moleculaire mechanismen, de Sandviken Stradivarius of gewoon over koeien en paarden. Bedankt dat je me de kans gaf om te pionieren, om toch even ontdekkingsreiziger te kunnen zijn. Ik heb ontzettend veel geleerd van die ervaringen. Wie weet wat we allemaal nog meer in de CalScreener gaan stoppen!

Dr Knight, dear Gwen, thank you for our fun weekly Thursday meetings. I have learned so much from you, from technical skills like R coding to life as a woman in STEM. Your enthusiasm for science and ability for interdisciplinary translation are an inspiration. During the last few years, you essentially turned into my third supervisor. I am happy that you are a member of my PhD committee and will see me out until the very end.

Mehri, het wordt vaker gezegd, maar zonder jou was dit boekje daadwerkelijk nooit tot stand gekomen. Bedankt voor jouw uitzonderlijke aandacht voor elk detail, ons vele contact tijdens de vroege COVID maanden en vooral het doen van talloze CalScreener experimenten en neuskweken. Het was ontzettend veel werk, maar het heeft tot mooie resultaten geleid.

Prof. Lindsay, dear Jodi, thank you for all the nice chats and encouragement, particularly during our last meetings in London and Utrecht last year. I appreciate the extra work you put in to make my thesis a success. How wonderful that you will be a member of my PhD committee, I am looking forward to it!

Graag wil ik Prof.dr. Pasmans, Prof.dr. Kluytmans, dr. Broens en de overige leden van de promotiecommissie bedanken voor de kritische beoordeling van mijn proefschrift en hun deelname in de promotiecommissie.

The MACOTRA study group: Adam, Anaïs, Anneke, Antoni, Arya, François, Gérard, Gwen, Jean-Philippe, Jodi, Leo, Mehri, Mirjam, Sake. It was great to do my PhD while being fostered by such a great group of researchers from so many different fields. And how wonderful that we could combine our meetings with a London pub crawl or a French cooking class! Thank you for the great chats, meetings, discussions and your critical reviews of our papers. I learned a lot from all of you!

De *S. aureus* groep: Corné, Deborah, Joop, Kirby, Mehri, Michèle, Nicole, Rofian en Susan. Bedankt voor jullie hulp in het lab en daarbuiten. Met het delen van kritische ideeën konden we elkaar altijd weer verder helpen. Rofian, Andi, double dr. Sultan, thank you for your introduction to the labwork, the CalScreener, biofilm and soto. We shared some good laughs and talks in my early PhD years and I enjoyed seeing you become a double dr. recently. I know you won't be able to attend my defence, but I appreciate the words of encouragement in this last phase!

Peter, Michella, Tanny, Annelies en andere collega's uit het LUMC. Bedankt voor het gezellige contact, jullie harde werk en de mogelijkheid een kijkje te nemen in jullie keuken! Het meekijken tijdens de huidexperimenten maakte de resultaten veel inzichtelijker.

Michael David and Harald Seifert, thank you for the nice meetings and collaboration. I am very grateful for your guidance during the writing and revision stages of our ISAC paper. Many thanks to all other participants of the laboratory survey and webinar participants, without you this study would not have been successful.

Ik wil graag alle deelnemers aan de neusmicrobioom-studie van harte bedanken voor hun inzet. Zonder jullie was hoofdstuk 6 van dit proefschrift er nooit gekomen.

Anja, Anne, Anneloes, Bertrand, Chinmoy, Cynthia, Dimard, Elise, Jannette, Maarten, Manon, Michèle, Ramzy, Rixt, Tanmoy, Wilson en andere MMIZ-collega's, bedankt voor alle tips, discussies en praatjes tussendoor. Anja en Manon, ook jullie zijn bijna zover, zet hem op! Carla, Ger en Kevin, bedankt dat jullie altijd weer klaarstonden om ergens mee te helpen of iets te regelen. Ger, bedankt voor het idee om PVC-kaartjes te gebruiken bij onze experimenten, het heeft gewerkt! Jannette, from the beginning we shared a lot, the work in the lab as well as the troubles at home. Also you will be defending your thesis soon, and I hope we will finally have time afterwards to visit as many cat cafés as we can find. In the meantime, thank you for being my friend and paranympth!

Als student Diergeneeskunde is het niet vanzelfsprekend om de overstap naar het onderzoek te maken. Ik wil dan ook iedereen bedanken die ervoor gezorgd heeft dat mijn voorzichtige stapjes in de onderzoekswereld zich ontwikkelden tot een zelfverzekerde sprong. Allereerst mijn mentor Herman Jonker, die me de moed gaf om eens met deze of gene hoogleraar te gaan kletsen. Daarnaast ook Gerrit Koop, onder wiens enthousiaste begeleiding ik voor het eerst kennis maakte met de klonale epidemiologie van *S. aureus*. Joe Rubin, Michelle and the other Rubidoos, thanks for a great time in Saskatoon, I absolutely loved it! En natuurlijk alle medewerkers van het KLIF, bij jullie heb ik mijn eerste echte stappen in het onderzoek mogen zetten! Jaap, bedankt voor jouw enthousiasme en het kritische commentaar op mijn eerste artikelen. Ik sta tijdens het schrijven nog regelmatig stil om eens even te bedenken wat ik nu eigenlijk bedoel met een stukje tekst! Els, leuk je weer te zien, dit keer als opponent! Joost, bedankt voor de fijne begeleiding, jij had altijd tijd om vragen te beantwoorden of iets nóg uitgebreider uit te leggen. Leuk dat we weer collega's zijn!

Inmiddels ben ik aan de slag bij het Centrum Z&O van het RIVM. Teekies: Hein, Miriam, Marieta, Manoj, Ankje, Robert, Marieke, Clara, Sara, Laura en Lukas, en alle andere (D&V) collega's: bedankt voor de warme ontvangst. We zijn goed begonnen, de eerste week bij jullie stond ik alweer met mijn voeten in de 'klei'.

Leuk dat ik af en toe weer een beetje dierenarts mag zijn. Ik ben benieuwd naar alle avonturen die jullie voor me in petto hebben!

Lieve Annieka, Christie, Elise, Evelien, Femmy, Jette, Joost, Lis, Marjolein, Matthijs, Michiel, Nienke, Roos en Sanne. Werk is veel leuker als je achteraf de (smerige) verhalen kan delen met je vrienden. Bedankt voor alle gezellige en chaotische weekendjes, dagjes Efteling, het kamperen, de vakanties en de talloze online en real-life spelletjesavonden. Jullie zijn leuk! Ook bedankt voor jullie medewerking aan mijn onderzoeksprojecten, in de vorm van poepjes of anderszins. Ik waardeer het enorm! Jette, bedankt voor het delen van jouw promotieverhalen, gedeelde smart maakt halve smart? Lis, jij bent de beste! Femmy, jij kent mij soms beter dan mezelf. De afgelopen jaren hebben we vele uren aan de telefoon gehangen om onze werkgerelateerde en andere perikelen te delen. Bedankt voor de motivatie. Ik ben heel blij dat je me ook bij de laatste horde wil helpen als mijn paranimf!

Tim, Gina, Gloria, Shawna, Finn and little Jack. Unfortunately, I wasn't able to cure MRSA, but your questions about my research were always very appreciated. Next summer I will have plenty more stories to share! Thank you for welcoming me into your family. I am looking forward to all the fun times to come.

Yvonne en Ruud, jullie hebben altijd veel belangstelling getoond voor al mijn bezigheden. Het is goed om te weten dat er zulke fijne mensen achter je staan. Tom en Marc, het is altijd leuk om te kletsen over reizen of te horen in welke musea of restaurants jullie nu weer zijn geweest, bedankt voor de gezelligheid! Tom, jij voorziet me al jaren van (meestal gewenst) advies en ik hoop dat je dat nog heel lang blijft doen. Marc, ook jij bent bijna klaar, succes met de laatste loodjes! Renée, wat er ook gebeurt, wij zijn voor altijd zusjes.

Pap en mam, gezien de gevulde boekenkasten thuis en jullie aanmoediging om altijd weer nieuwe dingen te ontdekken, is het niet gek dat ik in de wetenschap terecht gekomen ben. Ook doorzettingsvermogen hebben jullie mij al vroeg aangeleerd. Het is dan ook grotendeels aan jullie te danken dat ik zover gekomen ben. Helaas is er de laatste jaren niet zoveel tijd en energie voor geweest, maar ik hoop dat we de komende jaren nog veel samen mogen leren en ontdekken, te beginnen met een grote reis naar Canada!

Andrew, my dearest Canadian, even though my PhD has only been a small detail on our journey together, you have helped me out in so many ways. Even without always fully understanding why, you willingly gave me access to your English vocabulary and grammar, thermodynamics knowledge and last-but-not-least your nose. I feel very lucky that you crossed the seas to be with me. Thank you for standing by my side through the past years and those to come!

Curriculum vitae

Valérie Baede was born on July 13th 1990 in Geldrop, the Netherlands. She finished her secondary education (VWO) from Pleincollege Sint-Joris in Eindhoven in 2008. After completing the first year of the bachelor degree in Biology, she was admitted to the Faculty of Veterinary Medicine of Utrecht University to start her bachelor degree in Veterinary Medicine in 2010. She wrote her bachelor thesis on the molecular epidemiology of *S. aureus* in small ruminants. She continued her master degree in Farm Animal Health and Public Health at Utrecht University. In 2014, she completed the MSc Honours Programme of the Faculty of Veterinary Medicine with research on extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* in companion animals at the Department of Immunology and Infectious Diseases, upon which she received the Diploma in Veterinary Research. For her minor in One Health, she attended elective courses on advanced veterinary epidemiology, mathematical modelling of infectious diseases and global health & tropical medicine. Additionally, she completed a research internship on antimicrobial susceptibility of *Brachyspira* spp at the Western College of Veterinary Medicine, University of Saskatchewan in Canada. As a student board member of the veterinary study association Archaeopteryx, she organised and attended events focused on One Health, zoonoses and wildlife health in the Netherlands and abroad. In 2017, she started her PhD at the department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam under supervision of prof. dr. Margreet Vos and dr. Willem van Wamel. In February 2023, Valérie started as a researcher at the National Institute for Public Health and the Environment to work on wildlife and vector-borne zoonoses.

List of publications

Baede, V. O., Voet, M. M., van der Reijden, T. J. K., van Wengen, A., Horst - Kreft, D. E., Lemmens - den Toom, N. A., Tavakol, M., Vos, M. C., Nibbering, P. H. & van Wamel, W. J. B. The survival of epidemic and sporadic MRSA on human skin mimics is determined by both host and bacterial factors. *Epidemiology and Infection* **150**, e203 (2022). doi: [10.1017/S0950268822001765](https://doi.org/10.1017/S0950268822001765)

Baede, V. O., Barray, A., Tavakol, M., Lina, G., Vos, M. C. & Rasigade, J.-P. Nasal microbiome disruption and recovery after mupirocin treatment in *Staphylococcus aureus* carriers and noncarriers. *Scientific Reports* **12**, 19738 (2022). doi: [10.1038/s41598-022-21453-4](https://doi.org/10.1038/s41598-022-21453-4)

Baede, V. O., Tavakol, M., Vos, M. C., Knight, G. M. & van Wamel, W. J. B. Dehydration Tolerance in Epidemic versus Nonepidemic MRSA Demonstrated by Isothermal Microcalorimetry. *Microbiology Spectrum* **10**, e0061522 (2022). doi: [10.1128/spectrum.00615-22](https://doi.org/10.1128/spectrum.00615-22)

Baede, V.O., David, M. Z., Andrasevic, A. T., Blanc, D.S., Borg, M., Brennan, G., Catry, B., Chabaud, A., Empel, J., Enger, H., Hallin, M., Ivanova, M., Kronenberg, A., Kuntaman, K., Rhod Larsen, A., Latour, K., Lindsay, J. A., Pichon, B., Santosaningsih, D., Schouls, L. M., Vandenesch, F., Werner, G., Žabicka, D., Žemličková, H., Seifert, H. & Vos, M.C. MRSA surveillance programmes worldwide: moving towards a harmonised international approach. *International Journal of Antimicrobial Agents* **59**, 106538 (2022). doi: [10.1016/j.ijantimicag.2022.106538](https://doi.org/10.1016/j.ijantimicag.2022.106538)

Baede, V. O., Broens, E. M., Spaninks, M. P., Timmerman, A. J., Graveland, H., Wagenaar, J. A., Duim, B. & Hordijk, J. Raw pet food as a risk factor for shedding of extended-spectrum beta-lactamase-producing Enterobacteriaceae in household cats. *PLoS One* **12**, e0187239 (2017). doi: [10.1371/journal.pone.0187239](https://doi.org/10.1371/journal.pone.0187239)

Baede, V. O., Wagenaar, J. A., Broens, E. M., Duim, B., Dohmen, W., Nijse, R., Timmerman, A. J. & Hordijk, J. Longitudinal Study of Extended-Spectrum- β -Lactamase- and AmpC-Producing Enterobacteriaceae in Household Dogs. *Antimicrobial Agents and Chemotherapy* **59**, 3117–24 (2015). doi: [10.1128/AAC.04576-14](https://doi.org/10.1128/AAC.04576-14)

PhD portfolio

Name: Valérie O. Baede
Erasmus MC Department: Medical Microbiology and Infectious Diseases
Promotor: Prof. dr. Margreet C. Vos
Copromotor: Dr. Willem J. B. van Wamel

PhD Training Courses

Microscopic Image Analysis: From Theory to Practice, MolMed	2018
Introduction in GraphPad Prism Version 7, MolMed	2018
Microsoft Access 2010: Basic, MolMed	2018
OpenClinica Training, DCT Erasmus MC	2018
The course on R, MolMed	2018
Basic course Rules and Organisation for Clinical researchers (BROK), NFU	2018
Microbiomics I, MolMed	2019
Writing Successful Grant Proposals, MolMed	2019
Scientific Integrity, Erasmus MC	2019
Advanced Biofilm Course, TU Delft	2019
Indesign CC, MolMed	2020
Photoshop and Illustrator CC, MolMed	2020
Excel VBA, MolMed	2020
OneNote, MolMed	2020
Gene expression data analysis using R, MolMed	2021
Leica Confocal course, OIC Erasmus MC	2021
Logframe: the basis for good project writing, RDO Erasmus MC	2021

Workshops and seminars

MMIZ Research meetings, weekly	2017-2021
MMIZ Journal clubs, weekly	2017-2021
MMIZ R&D Science Day	2017,2019
MACOTRA Consortium Meetings, biannual Rotterdam, Lyon (F), London (UK)	2017-2020
Shopping for Funding, RDO Erasmus MC	2017
AMR symposium, Netherlands Centre for One Health	2017
RESIST, AMR modelling workshop, LSHTM, London (UK)	2018
Annual meeting, Netherlands Centre for One Health	2018
Evolutie of Revolutie, NWO & ZonMw, Den Haag	2019

Interventions to Reduce the Development and Transmission of AMR, JPIAMR (online)	2020
Regional & National MRSA Surveillance Programmes Worldwide, ISAC (online)	2021
<i>Oral presentation</i>	

International conferences

5th International One Health Congress, Saskatoon, Canada	2018
18 th ISSSI, Copenhagen, Denmark	2018
<i>Poster presentation</i>	
29th ECCMID, Amsterdam	2019
<i>Mini-oral ePoster Session</i>	
31th ECCMID, online	2021
<i>ePoster</i>	
<i>Oral presentation (travel grant)</i>	
<i>Oral presentation (travel grant)</i>	

Teaching

MGZ Community onderwijs	2018-2019
Thema 2.B I&I Vaardigheidsonderwijs	2018-2021

