Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Epidemiological, phenotypic and molecular profile of *Staphylococcus aureus* causing bacteraemia in children younger than 5 years in Manhiça district, Mozambique, 2001- 2019

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TESE PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE EM BIOLOGIA CELULAR E MOLECULAR

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To God

To my dear father (In memoriam) and mother

To myself

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Resumo

A bacteremia estafilocócica, em particular a associada a *Staphylococcus aureus* (SAB), é uma das infeções da corrente sanguínea mais comuns em todo o mundo, mas ainda pouco caracterizada em Moçambique, particularmente ao nível da caracterização epidemiológica e das estirpes bacterianas associadas, informação essencial para definir estratégias de controlo e prevenção eficazes. Neste Doutoramento procurou-se descrever a epidemiologia de SAB e caracterizar *S. aureus* isolados em hemoculturas de crianças menores de 5 anos internadas no Hospital Distrital de Manhiça, Moçambique, ao longo de duas décadas (2001-2019). A incidência de SAB foi calculada a partir do sistema de vigilância demográfica do distrito da Manhiça. Entre 2001 e 2019, 8% (3.197/41.891) destas crianças apresentaram bacteremia, 12% das quais correspondendo a SAB. A incidência global de SAB foi de 56,1 episódios/100.000 crianças-anos em risco (CYAR), sendo mais elevada entre os neonatos (589,8 episódios/100.000 CYAR). A incidência de SAB diminuiu significativamente, de 322,1 para 12,5 episódios/100.000 CYAR entre 2001 e 2019. A análise de suscetibilidade aos antimicrobianos revelou uma elevada frequência de estirpes multirresistentes - MDR (25%, 85/336), mas reduzido número de *S. aureus* resistentes à meticilina - MRSA (5% 16/336). Detetou-se elevada frequência de resistência à penicilina (90%, 304/336), um antibiótico prescrito empiricamente (em combinação com a gentamicina) para doentes com suspeita de doença bacteriana invasiva. A mortalidade hospitalar por SAB foi de 9% (31/332), sendo significativamente associada a infeções por estirpes MDR (p = 0,043) e MRSA (p = 0,006). A tipificação molecular por *Sma*I-PFGE, *spa typing* e MLST revelou elevada diversidade de clones de *S. aureus* circulantes, com predominância dos complexos clonais CC5 (17%, 58/336) e CC8 (16%), seguidos por CC15 e CC1 (11% cada). A linhagem CC152, inicialmente detetada em 2001, ressurgiu em 2010 e tornou-se predominante no restante período de vigilância, enquanto outros CCs (CC1, CC5, CC8, CC15, CC25, CC80 e CC88) diminuíram ao longo do tempo. As 16 estirpes MRSA detetadas pertencem aos clones t064-ST612/CC8-SCC*mec*IVd (11/16), t008-ST8/CC8-SCC*mec*NT (4/16) e t5351-ST88/CC88-SCC*mec*IVa (1/16). Os genes *lukSF-PV*, que codificam para a leucocidina de Panton-Valentine foram detetados em 44% (147/336) das estirpes, predominando nos CC22 (100%), CC152 (97%) e CC121 (85%). Nenhuma das estirpes MRSA apresentou estes genes. A produção de biofilme foi frequentemente detetada para estirpes das linhagens emergentes CC152 e CC121 (80%, 52/65), sendo esta produção particularmente forte nas estirpes CC152. Os ensaios de infeção de *Galleria mellonella* com estirpes representativas evidenciaram um elevado potencial de virulência das estirpes clínicas testadas, com variações dentro e entre os CCs analisados. Apesar da incidência decrescente de SAB na nossa população, a mortalidade associada é ainda elevada, possivelmente relacionada com a infeção por estirpes MDR ou MRSA não tratáveis com os antibióticos comummente disponíveis. Estes resultados indicam uma necessidade urgente da revisão da pauta antibiótica empírica actual em Moçambique, tal como suportado pela actual preocupação relativa a resistência antimicrobiana. Adicionalmente, deve ser priorizado o desenho e implementação de medidas preventivas direcionadas para clones associados a SAB emergentes, incluindo o desenho de abordagens para a deteção precoce e tratamento de crianças com infeções potencialmente letais.

Palavras-chave: *Staphylococcus aureus*, bacteremia, pediátrico, epidemiologia, tipificação molecular, Moçambique

Abstract

Staphylococcus aureus bacteraemia (SAB) is one of the most common bloodstream infections globally. Data on SAB epidemiology and associated bacterial lineages are scarce in Mozambique but needed to define effective preventive and control strategies.

This doctoral thesis aimed to describe the epidemiology of SAB and characterization of associated *S. aureus* isolates from blood cultures among children aged under 5 admitted to the Manhiça District Hospital (MDH), Manhiça, Mozambique over two decades (2001-2019). The SAB incidence was calculated from the demographic surveillance system established in our setting. Between 2001 and 2019, 8% (3,197/41,891) of all children admitted at MDH had bacteraemia, 12% of which corresponded to SAB. The overall SAB incidence was 56.1 episodes/100,000 children-years at risk (CYAR) and was highest among neonates (589.8 episodes/100,000 CYAR). SAB declined significantly over the two decades of surveillance (322.1 to 12.5 episodes/100,000 CYAR between 2001 and 2019). The antimicrobial susceptibility analysis of the SAB-related *S. aureus* revealed high rates of multidrug-resistant (MDR) strains (25%, 85/336) and a low number of methicillin-resistant *S. aureus* (MRSA) strains (5%, 16/336). High frequencies of resistance were observed for penicillin (90%, 304/336), an antibiotic empirically prescribed (together with gentamicin) for patients admitted with suspected invasive bacterial disease. In-hospital mortality by SAB was 9% (31/332), significantly associated with infections by MDR ($p = 0.043$) and MRSA strains ($p = 0.006$). Molecular typing by S*maI*-PFGE, *spa* typing and MLST revealed highly diverse circulating *S. aureus* clones with a predominance of clonal complexes CC5 (17%, 58/336) and CC8 (16%), followed by CC15 and CC1 (11% each). CC152, initially detected in 2001, re-emerged in 2010 and became predominant throughout the remaining surveillance period, while other CCs (CC1, CC5, CC8, CC15, CC25, CC80, and CC88) decreased over time. The 16 MRSA strains detected belonged to clones t064-ST612/CC8-SCC*mec*IVd (11/16), t008-ST8/CC8-SCC*mec*NT (4/16) and t5351-ST88/CC88-SCC*mec*IVa (1/16). The Panton-Valentine leucocidin (PVL) encoding genes, *lukSF-PV*, were carried by 44% (147/336) strains, predominantly among CC22 (100%), CC152 (97%) and CC121 (85%). All MRSA were *lukSF-PV* negative. The strains from the emergent lineages CC152 and CC121 were frequent biofilm producers (80%, 52/65), which was stronger for CC152. The infection assays of *Galleria mellonellla* with representative strains revealed high virulence potential of all SAB-related *S. aureus* clinical strains tested compared to a reference strain, with variation within and between the CCs analyzed. The findings from this Thesis revealed that despite the declining rate of SAB in our population, the associated mortality rate is still high, possibly related to infection by MDR and MRSA strains untreatable with the current empiric antibiotic therapy. These findings suggest an urgent need to review the current guidelines of empiric antibiotic treatment in Mozambique, as supported by the current concern regarding the burden of antimicrobial resistance. In addition, the design and implementation of preventive measures targeting SAB-related emerging clones, including tools for early recognition and treatment of children at risk of life-threatening disease must be urgently addressed.

Keywords: *Staphylococcus aureus*, bacteraemia, paediatric, epidemiology, molecular typing, Mozambique.

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AST - antimicrobial susceptibility testing
CA-MRSA - community-acquired methicillin-resistant *Staphylococcus aureus*
CC - clonal complex
ccr - cassette chromosome recombinase
CIP - ciprofloxacin
CISM - Manhiça Health Research Centre (*Centro de Investigação em Saúde de Manhiça)*
CFR - case fatality rate
CHAMPS - Child Health and Mortality Prevention Surveillance
CHL - chloramphenicol
CLSI - Clinical Laboratory Standards Institute, Illinois, USA
CLID - clindamycin
CYAR - children-years at risk
DAP - daptomycin
DNA - deoxyribonucleic acid
DSS - Demographic Surveillance System
ETs - exfoliative toxins
ERY - erythromycin
FOX - cefoxitin
GEN - gentamicin
HA-MRSA - hospital-acquired methicillin-resistant *Staphylococcus aureus*
HDSS - Health and Demographic Surveillance System
HIV - human immunodeficiency virus
HVR - hypervariable region
LOS - length of stay
MLS_B - macrolide, lincosamide and streptogramine
MIC - minimal inhibitory concentration
MCH - Maputo Central Hospital, Mozambique
MDH - Manhiça District Hospital, Mozambique
MDR - multidrug resistant
MGEs - mobile genetic elements
MLST - multilocus sequence typing
MRSA - methicillin-resistant *Staphylococcus aureus*
MSSA - methicillin-susceptible *Staphylococcus aureus*
Perm_ID - permanent identification number
PBPs - penicillin binding proteins
PCR - polymerase chain reaction
PEN - penicillin

PFGE - pulse field gel electrophoresis
PVL - panton-valentine leukocidin
QRDR - quinolone-resistance determining region
SAB - *Staphylococcus aureus* bacteraemia
SCC*mec* - Staphylococcal chromosomal cassette *mec*
SE - enterotoxins
SID - simpson´s index of diversity
SD - standard deviation
SLVs - single locus variants
spa - staphylococcal protein A
SSTIs - skin and soft tissue infections
ST - sequence type
SXT - trimethoprim/sulfamethoxazole
TCY - tetracycline
TMP - trimethoprim
TSST-1 - toxic shock syndrome toxin-1
VAN - vancomycin
VISA - vancomycin intermediate-resistant *S. aureus*
VNTR - variable-number tandem repeats
WGS - whole genome sequencing
WHO - World Health Organization

Units
bp - base pair
°C - degree Celsius
rpm - rotation per minute
min - minute
cm - centimetre
mL - millilitre
mM - micromolar
Mb - megabase
cfu - colony forming unit
h - hour
CI - confidence interval
µl - microliter
OR - odds ratio
IQR - interquartile range
p - *p*-value

Thesis outline

This Thesis describes the epidemiology and characterization of *Staphylococcus aureus* causing bacteraemia in children under 5 years of age admitted at the Manhiça District Hospital (MDH), Mozambique over two decades (2001 – 2019).

The Thesis is structured in five chapters. **CHAPTER 1 (General Introduction)** describes the Manhiça District, where the study was conducted, and existing health and research facilities, namely the Manhiça Health Research Centre (Centro de Investigação em Saúde de Manhiça) and the Health and Demographic Surveillance System. It also includes a general description of *S. aureus*, addressing virulence factors and antibiotic resistance mechanisms as well as globally disseminated clones, and the burden of *S. aureus* related bacteraemia (SAB). Finally, the general and specific objectives of this Thesis are presented. The Thesis main results are presented in the following three chapters as manuscripts, already published or submitted for publication. **CHAPTER 2 (Epidemiology and clinical presentation of community-acquired SAB in children under 5 years of age admitted to the MDH, Mozambique, 2001-2019)** describes the incidence, the clinical presentation and in-hospital mortality due to SAB, as well as the antibiotic prescription for patients with SAB, and comparison between patients infected by multiresistant strains and outcome. **CHAPTER 3 (Antimicrobial resistance and clonality of *S. aureus* causing bacteraemia in children admitted to the MDH, Mozambique, over two decades)** details the clonality of the *S. aureus* strains causing SAB in our setting as well as their antimicrobial resistance profiles and associated resistance determinants. **CHAPTER 4 (Exploring the virulence potential of *S. aureus* CC121 and CC152 lineages related to paediatric community-acquired bacteraemia in Manhiça, Mozambique)** evaluates the virulence potential of the most frequent and emergent *S. aureus* clonal lineages identified in our setting. These were tested for the presence of *lukSF-PV* genes, biofilm production and virulence potential in the *Galleria mellonella* infection model. **CHAPTER 5 (General Discussion and Conclusions)** addresses the main findings of this Thesis and their potential impact and implications for defining public health policies at the local and national level, particularly guiding the the clinicians for appropriate clinical management of the patients admitted to the hospital with SAB in the Manhiça District district and Mozambique, and concluding remarks.

CHAPTER 1 – Introduction

1.1. The Manhiça District: geography and health facilities

The Manhiça district is a rural area in the Maputo province in southern Mozambique, located 80 km north of Maputo City, Mozambique's capital (Figure 1.1). The district is located on a plain, surrounded by the Incomati river, covering an area of 2,380 km^2. The area has a subtropical climate, with a warm and rainy season (November to April) and a cool and drier season, during the rest of the year. The district has 15 health facilities, including the Manhiça District Hospital (MDH), the referral hospital in the district, equipped with laboratory, X-ray, admission wards, maternity, paediatric, surgery, nutritional services and others (Nhacolo *et al.*, 2021).

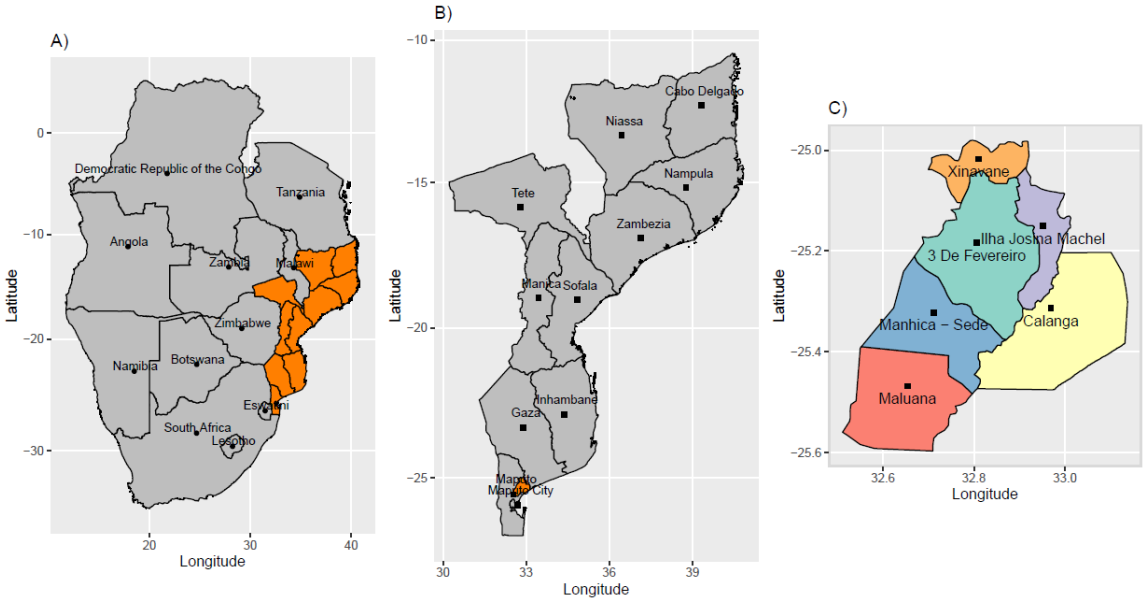

Figure 1.1. Map of the Manhiça district. (A) Location of Mozambique within part of the map of African continent. (B) Map of Mozambique depicting the 11 provinces. (C) Map of the Manhiça district showing each administrative post (n = 6). The provinces are the largest administrative divisions in Mozambique; subdivided into districts (each divided into administrative posts) (Figure prepared by Augusto Messa Jr, using R version 4.1.1, https://www.R-project.org/).

1.1.1. The creation of the Manhiça Health Research Centre (CISM)

The Manhiça Health Research Centre (*Centro de Investigação em Saúde de Manhiça* – CISM; https://www.cismmanhica.org/) was established in 1996 as a collaborative program between Spanish and Mozambican Governments to promote and conduct biomedical research on diseases representing a major morbidity and mortality burden for the local population (malaria, tuberculosis, diarrhea, HIV/AIDS, respiratory infections and invasive bacterial diseases), targeting primarily the most vulnerable population (children and pregnant women). In addition, the CISM aims to promote training in biomedical research for young Mozambican graduates from related fields to increase the critical mass of national scientists. CISM initially started producing baseline socio-demographic observational studies describing the patterns and trends of fertility, migration, and mortality in the area, together with basic descriptive studies on the epidemiology and burden of the most prevalent infectious diseases. CISM has widened its scope to include more in-depth molecular, immunological, and entomological studies, clinical trials and other intervention studies covering the major health problems of the country (Sacoor *et al.*, 2013).

Samples collected in the health facilities from specific studies are managed and archived using specific laboratory information system. The samples are identified with a labelled sticker, which includes a barcode-based unique identifier called NIDA (Sample Identification number), and the processing results are stored in the centralized databases. CISM has distinct laboratories for samples processing, namely immunology, bacteriology, mycobacteriology, molecular biology, parasitology, hematology, and biochemistry lab. The bacteriology lab is equipped to perform culture, isolation, identification and antimicrobial susceptibility testing of important bacterial pathogens from a variety of clinical samples. The molecular biology lab is equipped to perform real time and conventional PCR, sequencing (Illlumina sequencer recently installed), and bead-based immunoassay (Luminex xMap technology).

1.1.2. Implementation of the Health and Demographic Surveillance System (HDSS) in the Manhiça District

Since its conception, CISM has been conducting a continuous Health and Demographic Surveillance System (HDSS) established in the Manhiça district (Nhacolo *et al.*, 2021) that documents vital events (pregnancy, birth and deaths) and migrations which initially covered 500 km^2 (old area). In 2014, the study area was extended by 1,880 km^2 (new area) and actually covers the entire district (2,380 km2) (Nhacolo *et al.*, 2021; Sacoor *et al.*, 2013). The objective of the HDSS is to provide a platform for conducting biomedical research on local priority diseases described above. To update the Demographic Surveillance System (DSS) data, each household is visited twice per year to collect data on pregnancies, pregnancy outcomes, migration, socio-economic conditions, and other variables at individual and household levels. Each person living in DSS area is issued a printed unique permanent identification number (Perm_ID) card (Sacoor *et al.*, 2013). Since 2011 all the data are collected and entered in a paper free software - Open Health Demographic System, installed on tablets and transferred to a central server at CISM.

In addition to collecting demographic and socio-economic data among all the population living in the surveillance area, CISM runs a 24-hours hospital-based morbidity surveillance for children under 15 years in seven out of the fifteen health facilities of the district. For this, each time that a child visits the outpatient or inpatient services, a specific form is filled-in with health data, clinical examinations, routine laboratory basic tests, diagnosis, and treatment or referral undertaken. The form has a space to fill-in the Perm_ID from the card, so that all the demographic and illness events are linked to each child by this Perm_ID. These forms are brought to CISM's data centre, where they are double-entered in a specific database (Nhacolo *et al.*, 2021). These data enable accurate estimation of demographic and health indicators (mortality and fertility rates, among others), as well as tracking patient information, including the number of hospital visits, diagnoses, and treatment administered in during each visit.

1.1.3. Child Health and Mortality Prevention Surveillance (CHAMPS) Network

As part of morbidity surveillance, since 2016 the CISM joined the Child Health and Mortality Prevention Surveillance (CHAMPS) network that tracks the overall and definitive cause-specific mortality rates (stillbirth and under-5) in countries with high mortality burden in sub-Saharan Africa (Ethiopia, Kenya, Mali, Mozambique, Nigeria [since 2022], Sierra Leone, and South Africa) and South Asia (Bangladesh, and Pakistan [since 2023]) using an innovative approach of sample collections through minimally invasive tissue sampling (MITS) to investigate cause of deaths. For this, CHAMPS sites have established facility and community-based mortality notification systems, which aim to report potentially eligible deaths, defined as under 5-year-old children deaths and stillbirths in a defined catchment area within 24 - 36 hours. The CHAMPS sites procedures include minimally invasive tissue sampling (MITS), postmortem laboratory and pathology testing, verbal autopsy, and clinical and demographic data. Based on the results of CHAMPS procedures a final cause of death is determined by an expert review panel (paediatricians, obstetricians, epidemiologists, pathologists, microbiologists and other health-care providers) following the World Health Organization (WHO) International Classification of Disease (ICD-10) highlighting the underline, immediate and comorbidity cause of death. The definitive cause of death is primarly shared with the families of the deceased child, local and national authorities, and global stakeholders to inform important health interventions, research, and action to save young lives and probably contribute to accelerating the reduction of childhood death and disability in low-resource settings. In Mozambique, the CHAMPS activities are running in two catchement areas: the Manhiça and the Quelimane districts, located in Southern and Central Mozambique (Maputo and Zambézia Provinces), respectively (Dowell *et al.*, 2019; Salzberg *et al.*, 2019). Prelimary CHAMPS data ranked *Staphylococcus aureus* in the top five list of bacterial pathogens in the pathways of death events among neonates and children under 5 years old (Bassat *et al.*, 2023; Taylor *et al.*, 2020), suggesting the need of its prompt recognition and appropriate management.

1.1.4. HDSS Populational data and mortality rates among children

The HDSS expansion conducted in the Manhiça district in 2014 (new area) enumerated 72,494 individuals, which increased the study population from 97,496 inhabitants to a total of 169,990. The most recent complete round of data collection terminated in December 2019 for the entire district and reported 201,845 inhabitants (27,560 of which were children aged <5 years) living in 46,441 households (Nhacolo *et al.*, 2021). The mortality trend among children under 5 years old showed continuous decrease over time, from 132.3 deaths/1,000 live births in 1998 to 76.4 in 2013 and 33.4 in 2019. Despite the reduction observed, there was a slight increase in mortality, after a lower level in 2016 (Figure 1.2).

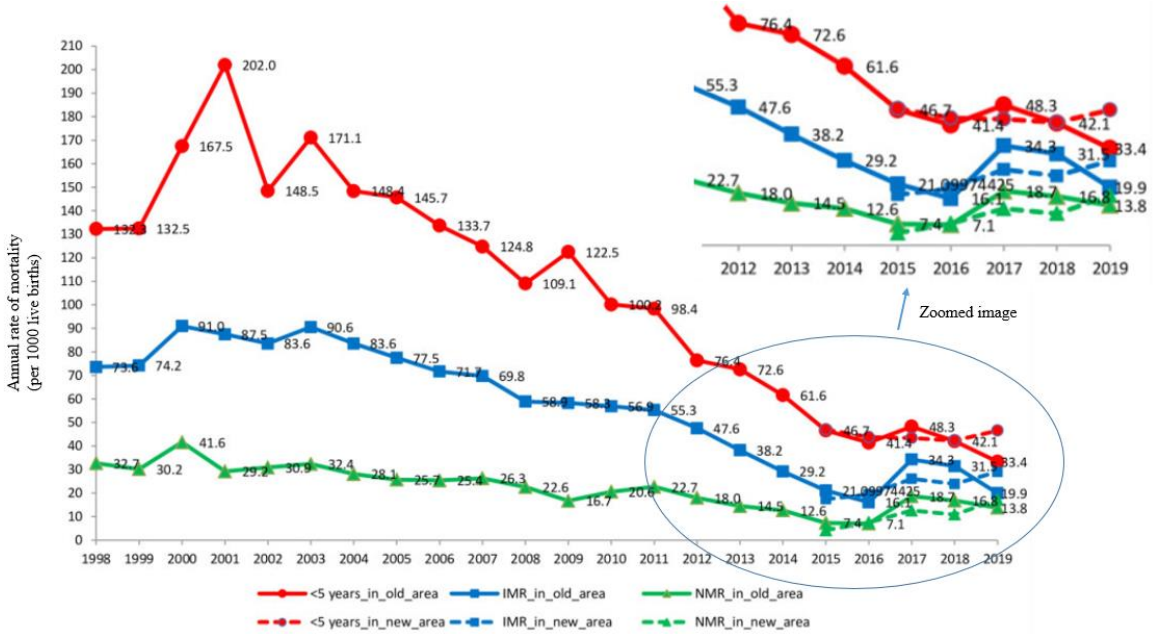

Figure 1.2. Mortality rates in neonates, infants and children <5 years old, Manhiça 1998–2019 by study area (old *vs.* new areas) (Adapted from Nhacolo *et al.*, 2021 under permission from Oxford University Press). The lines labelled <5 years_in_old_area and <5 years_in_new_area stand for the conventional mortality rate in children <5 years in old and new areas, respectively. The lines labelled IMR_in_old_area and IMR_in_new_area stand for infant mortality rate in old and new areas, respectively. The lines labelled NMR_in_old_area and NMR_in_new_area stand for neonatal mortality rate in old and new areas, respectively.

1.1.5. Outcomes of HDSS implementation in the Manhiça district

Our HDSS identified a perennial malaria transmission, mostly due to *Plasmodium falciparum* (Saúte *et al.*, 2003), and high HIV prevalence among the general adult population (40%) (González *et al.*, 2012), pregnant women (23.6%) attending the antenatal clinic (Menéndez

et al., 2008) and among sick children (25%) attending the hospital with moderate-to-severe diarrhoea (Acácio *et al.*, 2018). Some of the previous studies conducted in this HDSS contributed to guide the health authorities and decision makers in defining or adjusting health policies such as the introduction of Mozambique's expanded programme of immunization with different vaccines (*Haemophilus influenzae* type b, *Pneumococcus*, rotavirus) and the development of the concept of Intermittent Preventive Treatment for Infants (IPTi) that led to the WHO recommendation of this method as best practice for the control of malaria among infants (WHO, 2013).

1.2. Bacteraemia and *S. aureus*-related bacteraemia (SAB)

S. aureus bacteraemia (SAB), here defined as the isolation of *S. aureus* from blood culture (Garrine *et al.*, 2023a), is one of the most common bloodstream infections globally, being associated with higher mortality (29 – 63%) (Kaasch *et al.*, 2014) compared to bloodstream infections caused by other Gram-positive bacteria (Gijón *et al.*, 2016). The incidence of SAB is higher in the first years of life, lower throughout young adulthood, then rising gradually with advancing age (Tong *et al.*, 2015). Although SAB is clearly a global health problem, its epidemiology is only well described in high-income countries (Hassoun *et al.*, 2017; Bai *et al.*, 2022a, 2022b), with scarce data from low- and middle-income countries, including sub-Saharan Africa (Marchello *et al.*, 2019; Chukwumeze *et al.*, 2021; Berkley *et al.*, 2005; Groome *et al.*, 2012; Nielsen *et al.*, 2012; Abebe *et al.*, 2021; Ombelet *et al.*, 2022; Dramowski *et al.*, 2021). The few studies conducted in Africa have reported SAB incidences of 27/100,000 person-years among children <5 years in Kenya (Berkley *et al.*, 2005)**,** 26/100,000 person-years among children <13 years in South Africa (Groome *et al.*, 2012) and 6/1,000 person-years among children aged <5 in Ghana (Nielsen *et al.*, 2012). Recent reports showed *S. aureus* as a common pathogen among bacteraemic patients in Nigeria (Chukwumeze *et al.*, 2021), Ethiopia (Abebe *et al.*, 2021), Benin (Ombelet *et al.*, 2022) and South Africa (Dramowski *et al.*, 2021).

1.2.1. Burden of bacteraemia and SAB in Mozambique

In Mozambique, epidemiological investigation of infections caused by *S. aureus* is hampered by the lack of functioning microbiology laboratories covering the entire country and data on the disease burden is limited to few studies in Southern (Preziosi *et al.*, 2015; Sigaúque *et al.*, 2009) and Central Mozambique (Beira and Quelimane cities) (Kenga *et al.*, 2021; Van der Meeren *et al.*, 2014a). Data from the Manhiça district (Southern Mozambique) between 2001-2006 indicated that *S. aureus* was the third (12%) cause of bacteraemia among children aged < 15 years admitted at the Manhiça District Hospital (MDH) and the first cause of bacteraemia among neonates (39%), with incidence rates ranging from 101 (1 - <5 year old) to 178 (<1 year old) per 100,000 children-years at risk (CYAR) and associated mortality of 6% (Sigaúque *et al.*, 2009). Recent CHAMPS data ranked *S. aureus* as an important contributor in mortality among neonates (4%) and children (9%). Despite the high burden of SAB observed in our setting, detailed epidemiological studies on incidence and trend of invasive bacterial pathogens focused on invasive Nonthyphoidal *Salmonella* (iNTS) (Mandomando *et al.*, 2009, 2015), *H. influenzae* type b (Hib) (Sigaúque *et al.*, 2013), agents of invasive pneumococcal diseases (IPD) (Sigaúque *et al.*, 2018) and *Escherichia coli* (Mandomando *et al.*, 2020); with no reports on SAB epidemiological trends.

1.2.2. Management of Bacteraemia in Mozambique

According to the Mozambican national guidelines (Glisser, 1997), empirical antimicrobial therapy starts on admission for children with suspected bacterial infection using parenteral combination of penicillin/ampicillin plus gentamicin or chloramphenicol (less prescribed due to its toxicity, despite occasional use in case of antibiotic stockout), as described in Table 1.1. Specifically in our setting, antibiotic therapy is reassessed based on blood culture results and patient clinical evolution. Ceftriaxone is used for infections by multidrug resistant (MDR) strains (defined as showing resistance to three or more unrelated classes of antibiotics (Magiorakos *et al.*, 2012)) or for severe disease. Because the MDH is located in a rural setting with limited diagnostic tools or available second line antibiotics, patients requiring alternative treatment (e.g. infected by methicillin resistant *Staphylococcus aureus*-MRSA strains) or those not showing clinical improvement are referred to the Maputo

CentralHospital (MCH). Patient requiring transfusion (e.g. in the case of severe anemia) are also transferred to MCH, when blood supply is not available at MDH.

Table 1.1. Antibiotics frequently administered in Mozambique for treatment of children with suspicion of bacteraemia (Glisser, 1997).

Antibiotics administered	Criteria
PEN + GEN	Children aged > 2 years with suspected bacteraemia
CHL[a]	Children aged > 2 years with suspected bacteraemia
AMP + GEN	Children aged < 2 years with suspected bacteraemia or severe malnutrition
CRO	Infection by MDR strains or severe disease
VAN	Infection by MRSA
SXT	Prophylaxis against opportunistic infection and for pneumonia by *Pneumocystis cariini*

[a]Less prescribed due to its toxicity, despite occasional use in case of antibiotic stockout. PEN, penicillin; GEN, gentamicin; CHL, chloramphenicol; AMP, ampicillin; CRO, ceftriaxone; VAN, vancomycin; SXT, sulfamethoxazole-trimethoprim (co-trimoxazole); MDR, multidrug resistant; MRSA, methicillin resistant *S. aureus.*

1.3. The Genus *Staphylococcus* and staphylococci

Staphylococci are Gram-positive spherical bacteria with 0.5-1.5 µm in diameter, which form a 'bunch of grapes (staphyle') cluster that defines the genus. The name *Staphylococcus* was primarily introduced by Ogston in 1883, and a year later (1884), the genus *Staphylococcus* was formally described by Rosenbach (Götz *et al.*, 2006). The genus *Staphylococcus* belongs to the family of *Staphylococcaceae*, order of Caryophanales. To date, the genus comprises 64 species, which are able to colonize or infect human and multiple animal species (Parte *et al.*, 2020). The bacteria are characteristically non-spore-forming, non-motile, facultative anaerobic, catalase-positive, oxidase-negative that can grow in 10% NaCl (Foster & Geoghegan, 2015). Staphylococci present an ultrastructure and chemical composition of the cell wall similar to that of other Gram-positive bacteria, consisting of peptidoglycan, techoic acids, and proteins. In 1940, Fairbrother introduced coagulase production as a major differentiating factor for staphylococcal species (Fairbrother, 1940). The coagulase producers were termed coagulase-positive staphylococci (CoPS) and include *S. aureus*, *Staphylococcus intermedius*, *Staphylococcus pseudintermedius*, *Staphylococcus coagulans* among others; while those nonproducing were termed coagulase-negative staphylococci (CoNS) and include *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*,

Staphylococcus hominis, among many other species (Becker *et al.*, 2014). Within the large genus *Staphylococcus*, *S. aureus* is the most versatile species, equipped with a wide range of virulence factors, resistance determinants and presenting a high pathogenic capacity (Tong *et al.*, 2015).

1.3.1. The species *S. aureus*

S. aureus is a facultative anaerobic microorganism that can grow at a temperature range of 18°C - 40ºC, with colonies presenting golden or yellow pigmentation. This species is biochemically distinguishable from most other staphylococcal species based on positive results of coagulase, novobiocin susceptibility, mannitol fermentation, and presence of deoxyribonuclease activity. The molecular identification of *S. aureus* includes nucleic acid hybridization, PCR and sequencing analysis of ribosomal ribonucleic acid genes and other conserved genes (e.g. species specific *nuc* gene) (Tille, 2022). The *S. aureus* genome is constituted of a circular genome (~2.9 Mbp), plus an assortment of mobile genetic elements (MGEs) that include plasmids, bacteriophages, pathogenicity islands (PIs), chromosomal cassettes, and transposons. Virulence and antimicrobial resistance genes are found both in the chromosome and in MGEs and may be transferred among different strains, distinct staphylococci or even other Gram-positive bacteria throughout horizontal gene transfer, contributing to distinct phenotypic and genetic plasticity of this bacteria (Partridge *et al.*, 2018; Lindsay, 2019).

1.3.2. Colonization and infection

S. aureus colonizes the human and animal skin and mucosal surfaces, including nose, throat, vaginal and intestinal tract (Liu, 2009; Peton & Le Loir, 2014). The nasal cavity is the primary ecological reservoir of *S. aureus* and between 30% - 40% of human individuals are persistently colonized by this bacterium (Wertheim *et al.*, 2005), with higher frequency among younger children and immunocompromised patients (Hidron *et al.*, 2010; Thurlow *et al.*, 2020). The initial exposure of *S. aureus* to host tissues beyond the skin and mucosal surface is thought to trigger upregulation of virulence genes, and the success of infection which is commonly endogenous depends on the effective evasion of host defenses (Gordon

et al., 2021). The pathogen escapes from the host defenses by leaving the bloodstream with its high concentration of cellular and humoral immune defense mechanisms to invade organs and tissues (Gordon *et al.*, 2021). In addition to act as an extracellular pathogen, *S. aureus* has been also recognized as acting intracellulary, allowing bacterial persistence, protection from antibiotics, and evasion of immune defenses (Becker, 2018). On the other hand, it also presents specific mechanisms of evasion as the production of a range of cytotoxins that destroy phagocytes (leukocidins), as well as mechanisms that trigger phagocyte apoptosis, inhibit complement factors, promote agglutination and induce thrombi formation (Gordon *et al.*, 2021). The successful invasion of internal tissues by *S. aureus* causes infections ranging from skin and soft tissue infections (SSTIs) with encapsulated cutaneous abscesses in the dermis and subcutis (Becker, 2018) to more serious and complex diseases such as deep-seated infections (e.g. osteomyelitis), pneumonia, infective endocarditis and bacteraemia (Tong *et al.*, 2015). This last (bacteraemia) is considered one of the most serious clinical presentations of community-onset and nosocomial infections by *S. aureus* (Tong et al., 2015).

1.3.3. Virulence factors

S. aureus produces a wide array of virulence factors, which include a plethora of toxins, immune evasion factors, and several protein and non-protein factors that enable host colonization during infection (Gordon *et al.*, 2021). *S. aureus* toxins are categorized in three groups: membrane damaging toxins, toxins that interfere with receptors, and secreted enzymes (Otto, 2018). Important *S. aureus* toxins include the toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs), exfoliative toxins (ETs), the immune evasion proteins CHIPS and SCIN, and the Panton-Valentine leukocidin (PVL) (Ahmad-Mansour *et al.*, 2021).

1.3.3.1. Membrane damaging toxins

These toxins cause pore formation in the cytoplasmic membrane (cytolysis), leaking vital molecules and metabolites (Otto, 2014). Despite several *S. aureus* produced membrane-damaging toxins (hemolysins, bi-component leukocidins, and phenolsoluble modulins (PSMs)), only PVL and α-toxin (α-hemolysin) are able to promote apoptosis (Ahmad-

Mansour *et al.*, 2021).

Firstly, identified in 1932 (Panton & Valentine, 1932), PVL is a cytotoxin composed of two proteins, LukS-PV and LukF-PV, encoded by the *lukS-PV* and *lukF-PV* genes carried on bacteriophages (Saeed *et al.*, 2018). The LukSF-PV proteins act synergistically to create one hetero-octameric unit which binds to the human complement receptors of neutrophils, monocytes and macrophages creating pores in cell membranes with consequent cell membrane lysis and death (Melles *et al.*, 2006). Additionally, PVL also induces release of pro-inflammatory cytokines and nuclear factor-kappa B in neutrophils, with important role in necrotizing (Ma *et al.*, 2012). PVL is not routinely detected in the diagnostic microbiology laboratories. When performed, the screening tends to focus on MRSA strains, particularly community acquired-MRSA (CA-MRSA), with consequent underestimation of the PVL global carriage among *S. aureus* (Saaed *et al.*, 2018). Reported PVL incidence varies considerably (5% - 75%) (Hussain *et al.*, 2022), according to the circulating strains (Saeed *et al.*, 2018), being frequently associated with the emergence and spread of global multidrug resistant and virulent clones of *S. aureus* (ST8/USA300; ST1/USA400; and the "European" ST80) (Saeed *et al.*, 2018; Tristan *et al.*, 2007) and the emergent hypervirulent ST121 lineage in Africa, Asia and Europe (Rao *et al.*, 2015). A controversial relation between carriage of *lukSF-PV* genes and SSTIs or invasive diseases has been proposed by several studies (Darboe *et al.*, 2019; McGuire *et al.*, 2023; Shallcross *et al.*, 2013). PVL-carrying phages in *S. aureus* can be induced during antibiotic treatment with tobramycin, ciprofloxacin, co-trimozaxole, and β-lactams facilitating their transmission among the *S. aureus* population (Goerke *et al.*, 2004, 2006; Rolain *et al.*, 2009; Saeed *et al.*, 2018).

Another important toxin in this group is the α-toxin, a 33-kDa pore-forming toxin encoded by the chromosomal *hla* gene, being virtually produced by all strains and part of the *S. aureus* toxin 'core set' (Ahmad-Mansour *et al.*, 2021). It presents lytic activity against red blood cells and a series of leukocytes, except neutrophils. Upon cell-binding, α-toxin forms heptameric pores in the host cell plasma membrane, leading to cell lysis (Otto, 2014). This toxin affects the innate immune effector cells, stimulating a hyper inflammatory response characteristic of bacterial pneumonia, and disrupting epithelial and endothelial barriers (Powers *et al.*, 2012). The toxin has been also associated with osteomielite and bacteraemia

in experimental infection models with clinical *S. aureus* strains (Ahmad-Mansour *et al.*, 2021).

1.3.3.2. Toxins that interfere with receptors

The toxins of this group comprise the ones from the enterotoxin superfamily, which consists of staphylococcal enterotoxins (SEs), the staphylococcal enterotoxin-like (SELs) proteins, and the toxic shock syndrome toxin-1 (TSST1). SEs, which include SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SER, and SET, are well known by their abilities to cause food poisoning by interfering with intestinal function with consequent emesis and diarrhea (Otto, 2014**).** Those toxins are also referred as staphylococcal super antigens (SAgs), due to their ability to trigger T cell activation and proliferation without the need for antigen processing by allowing nonspecific interaction of the class II major histocompatibility complex MHC II with T cell receptors, stimulating hyper-inflammatory responses (Otto, 2014; X. Zhang *et al.*, 2017). The TSST1 is a 22-kD *S. aureus* superantigen toxin associated with toxic shock syndrome (TSS), a severe and fatal condition as consequence of releasing of IL-1, IL-2, TNF-α, and other cytokines (Spaulding *et al.*, 2013). However, the role of SAgs in bacteraemia and sepsis is still a matter of discussion (Oliveira *et al.*, 2018).

1.3.3.3. Secreted toxins and enzymes

Most *S. aureus* secreted enzymes are proteases which degrade host molecules or interfere with host metabolic and/or signaling cascades. Aureolysin, glutamyl endopeptidase, and the cysteine proteases staphopain A and B all interfere with complement factors, leading to evasion of complement-mediated bacterial killing (Otto, 2014). The exfoliative toxins specifically cleave the desmosomal cadherins of the superficial skin layers, leading to staphylococcal scalded skin syndrome (SSSS), a severe skin disease presenting with rash, blisters, and severe lesional damage of the skin (Ahmad-Mansour *et al.*, 2021). The staphylokinase and collagenase degrade fibrin clots and collagen facilitating bacterial penetration through the skin and muscle tissue (Otto, 2014). In addition, *S. aureus* produces two coagulases (staphylocoagulase and von Willebrand factor (vWF)) responsible for the formation of fibrin clots after binding to prothrombin and other plasma proteins, inhibiting

phagocytosis and causing abscess formation and adhesion of the pathogen to catheters during biofilm-associated infection (McAdow *et al.*, 2012).

1.3.4. Biofilm formation

A biofilm is a microbial community of cells embedded in a matrix of extracellular polymeric substances that they have produced; these aggregates can be attached to biotic or abiotic surfaces. Many human infections by *S. aureus* progress with involvement of biofilms or originate from biofilm-associated primary infections (Otto, 2018). As a frequent colonizer of the human skin, *S. aureus* can form biofilms on surgically implanted indwelling medical devices spreading into the bloodstream, producing serious infections, including endocarditis, prosthetic joint infections and bactereaemia (Idrees at al., 2021). Despite the association of biofilm with bacteraemia, some reports did not associate the biofilm production with poor clinical outcome of patients with SAB (Guembe *et al.*, 2018).

The main role of biofilm formation during infection is to protect the bacteria from phagocyte attacks, as the pathogen has the ability to skew the host immune response toward an anti-inflammatory state (Peng *et al.*, 2022). Staphylococcal biofilm formation develops in four main stages: attachment, accumulation/proliferation, maturation and biofilm dispersal (Moormeier & Bayles, 2017; Otto, 2018).

Attachment phase

For the biofilm formation on biotic materials, *S. aureus* attach to a surface through a variety of cell wall-anchored (CWA) proteins specific for different host matrix substrates (Moormeier & Bayles, 2017). One of most important family surface-expressed staphylococcal binding proteins are the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). These proteins consist, from the N- to C-terminus, of a signal peptide, a ligand-binding domain with characteristic repeat sequences, a cell wall-anchoring region, a membrane-spanning region, and a positively charged tail. At the C-terminus, the MSCRAMM proteins contain a conserved cell targeting motif (LPXTG motif) which have been implicated in binding host matrix components (e.g. fibronectin, fibrinogen, collagen and cytokeratin) to initiate cell adherence and/or biofilm development (Otto, 2018).

The most commonly known MSCRAMM are fibronectin-binding proteins (FnBPA and FnBPB), serine-aspartate repeat family proteins (SdrC, SdrD and SdrE), clumping factors (ClfA and CflB), collagen adhesion, protein A, plasmin-sensitive protein (Pls), SasG, ironregulated surface determinants (IsdA, IsdB, IsdC and IsdH) and bone sialoprotein (Bbp). On the other hand, the attachment of *S. aureus* to abiotic surfaces is mediated by electrostatic and hydrophobic interactions. Specific staphylococcal molecules as AtlE and wall teichoic acids (WTAs) have been implicated in the surface attachment of *S. aureus* (Idrees *et al.*, 2021).

Accumulation/proliferation

In the second stage of biofilm development, the microcolonies formed after attachment to a biotic or abiotic surface grow by proliferating in the presence of sufficient nutrient source, originating macrocolonies. In this stage *S. aureus* produces polymeric molecules of distinct chemical nature such as polysaccharides, proteins, and teichoic acids which contribute to biofilm matrix (Otto, 2018). The polymeric substances from dead cells, including extracellular DNA (eDNA) also contribute to the biofilm matrix (Foulston *et al.*, 2014). *S. aureus* produces exopolysaccharides, an important matrix component specifically implicated with biofilm formation. Among those, the polysaccharide intercellular adhesion (PIA) is one of the most important, responsible for electrostatic interactions with other surface polymers, and formation of the sticky extracellular biofilm matrix, in addition to providing increased resistance to antimicrobial peptides (e.g. reduced diffusion troughout the biofilm structure) and neutrophil phagocytosis. PIA biosynthesis is accomplished by the products of the *ica* (intercellular adhesion) operon, comprising the *icaA, icaD, icaB,* and *icaC* genes. On the other hand, in *vivo* and/or in *vitro* biofilm formation can be accomplished by *S. aureus* strains that do not harbor the *ica* locus, being intermediate by other proteins related to PIA (Nguyen *et al.*, 2020).

Maturation

At this stage, the biofilm is highly structured and a compact three-dimensional mushroom or tower structure is formed. A large number of channels around the macrocolonies are

constructed to provide increased surface area for nutrient exchange and waste removal, and promoting the biofilm cells dissemination to distal sites (Otto, 2018). This stage is supported by important surfaces proteins, including the Sas family of proteins, clumping factors, fibronectin-binding proteins, elastin-binding proteins, and protein AtlA (Moormeier and Bayles, 2017). These mature biofilms have diverse and unique metabolic structures that enable them to resist harmful environmental factors and stress drivers (Peng *et al.*, 2022).

Dispersal

Once the biofilm is fully matured, it disperses and releases the sessile cells which are ready to repopulate either the same site (primary site) or a new one (secondary). The *S. aureus* biofilm dispersal is regulated by four different genes of the accessory gene regulatory (agr) system, including *agrA, agrB, agrC and agrD*. The Agr-regulated dispersal of biofilm is brought about by the induction of different proteases and PSMs, which act as surfactants (Idrees *et al.*, 2021; Otto, 2018). The *agr* system regulation also affects the expression of virulence determinants to the state of infection contributing for nutrient acquisition, immune evasion, and preventing the trigger of immune responses (Gordon *et al.*, 2021).

1.3.5. Antibiotic resistance in *S. aureus*

S. aureus infections are particularly problematic due to frequent occurrence of antibiotic resistance, among which MRSA and MDR strains are the most clinically important (Kern & Rieg, 2020; Turner *et al.*, 2019; Tacconelli *et al.*, 2018). Infections by MRSA and MDR strains are accompanied by increased morbidity, length of hospital stay (LOS) and mortality compared to methicillin-susceptible *S. aureus* (MSSA)/non-MDR (Ippolito *et al.*, 2010). In 2017, the WHO included MRSA and vancomycin-resistant *S. aureus* in the high-priority list of antibiotic-resistant bacteria to support research and development of new antibiotics (Tacconelli *et al.*, 2018). Recently, *S. aureus* was ranked as the second pathogen associated with deaths by bacterial infections worldwide, responsible for 250,000 deaths attributable to antibiotic resistance in 2019, in which MRSA caused more than 100,000 deaths and 3.5 million disability-adjusted life-years (Murray *et al.*, 2022). Antibiotic resistance exhibited by *S. aureus* results either from determinants encoded on the chromosome or from additional

resistance genes acquired by horizontal transfer of individual genes or resistance islands from other *S. aureus* strains as well as from other bacterial species (Partridge *et al.*, 2018; Vestergaard *et al.*, 2019), contributing for resistance to multiple antibiotics classes (Pantosti *et al.*, 2007).

1.3.5.1. Resistance to ß-lactams

The β-lactam drugs act by inhibiting the bacterial cell-wall biosynthesis at the level of peptidoglycan synthesis, the main structural component of the cell wall. The peptidoglycan synthesis occurs through transglycosylation and transpeptidation reactions, carried out by the penicillin binding proteins (PBPs), which are the specific target of β-lactams. Briefly, the β-lactams inhibit the transpeptidation step of cell-wall biosynthesis by acting as substrate analogs of the D-Ala–D-Ala peptidoglycan side chain upon which PBPs act. A long-lived covalent acyl-enzyme complex forms between the β-lactam and the nucleophilic serine of the PBP active site, inhibiting cell-wall transpeptidation. Deacylation of this complex, as occurs during normal turnover, is impeded because the region of the active site that accommodates the deacylating acceptor moiety or a potential hydrolyzing water molecule is occupied by the β-lactam ring structure. Regeneration of the PBP is so slow relative to cell division that the enzyme is effectively irreversibly inactivated. The consequential loss of cell-wall cross-linking leads to defective cross-wall formation during cell division, followed by cell death (Peacock & Paterson, 2015).

S. aureus was susceptible to penicillin for treatment of related infections in the 1940s. However, nowadays more than 90% of *S. aureus* strains are resistant to this antibiotic, rendering penicillin essentially useless to treat these infections (Peacock & Paterson, 2015). The resistance to penicillin is due to the production of the penicillinase BlaZ, an extracellular enzyme (class A β-lactamase), that hydrolyzes the amide bond of the β-lactam ring of penicillin and ampicillin, yielding an inactive molecule. The β-lactamase BlaZ is encoded by the *blaZ* gene under the control of two regulatory genes, *blaI* and *blaR1*. The *blaR1* product, BlaR1, is a sensor-transducer which in the presence of penicillin or a structurally similar molecule, initiates a cascade of events leading to enhanced penicillinase expression (Foster, 2017). Plasmids encoding penicillinase production may also carry additional resistance

determinants against other antibiotic agents (Lowy, 2003).

Methicillin was the first semisynthetic penicillinase-resistant β-lactam clinically available, marketed in 1959 (as Celbenin) in response to the emergence of penicillin resistance (Peacock & Paterson, 2015). However, only one year after the introduction of this antibiotic the first MRSA strains were described (Barber, 1961; Jevons, 1961). In the following years MRSA strains emerged in many countries, including those where methicillin was not available, being now ubiquitous all over the world (Lee *et al.*, 2018). Unlike the resistance of *S. aureus* to penicillin, methicillin resistance is not mediated by a plasmid-borne β-lactamase, instead it is mediated by the *mec* genes (*mecA* or *mecC*), which are located in a staphylococcal chromosomal cassette, SCC*mec*. The *mec* genes encode an alternative penicillin-binding protein, designated PBP2a or PBP2', in which the active-site serine is significantly less accessible to β-lactams when compared to the native PBPs. This lower accessibility is originated on the active-site serine location in a narrow-extended cleft that has a lower affinity for beta-lactams (and other penicillin-derived antibiotics). Therefore, PBP2a continues to catalyze the synthesis of the bacterial cell wall even in the presence of ß-lactam antibiotics (Stapleton & Taylor, 2002), assuring the transpeptidation reaction of the peptidoglycan synthesis whereas transglycosylation is ensured by the transglycosylase domain of the native PBP2 that remains active (Pinho *et al.*, 2001).

The acquisition of the cassette containing the *mec* gene (SCC*mec*) not only confers resistance to methicillin but also to all the antibiotics belonging to the ß-lactam classes except for the last generation of cephalosporin β-lactams (i.e., ceftaroline, ceftobiprole) (Lee *et al.*, 2018). In terms of structure, the SCC*mec* elements contain a *mec* gene complex comprising of *mecA* and its regulatory genes *mecI* and *mecR1*, a cassette chromosome recombinase (*ccr*) gene complex containing one or two site-specific recombinase genes responsible for the movement of the SCC*mec*, insertion sequences, and typically three J (junk) regions which encode important functions such as resistance to additional antibiotics and to heavy metals.

The transcription of *mecA* is induced in the presence of β-lactams by a signal transduction system encoded by the *mec* gene complex, the MecR1 sensor (an integral-membrane zinc dependent sensor) and the MecI (a transcriptional repressor), whose determinants are located adjacent to *mecA*. The MecR1 sensor detects the presence of β-lactams via its extracellular

penicillin-binding domain (PBD). Upon β-lactam-mediated acylation, a conformational change induces autocatalytic cleavage of the intracellular sensor domain, which in turn, either directly or indirectly, leads to cleavage of MecI. This process impedes MecI binding to the promoter region and induces *mecA* expression and methicillin resistance. As MecR1 and MecI are turned over by signal transduction, their synthesis is also induced by β-lactam signaling, allowing return to a basal state when β-lactams are removed (Peacock & Paterson, 2015). The *mec* regulator genes may be intact, partially or totally deleted. The different combinations of composition and structure of the SCC*mec* element are the basis for their classification into different cassestte types, as detailed below (section 1.4.1.4 SCC*mec* typing).

1.3.5.2. Resistance to aminoglycosides

Aminoglycosides are bactericidal antibiotics and are synergistic with other antibiotics (e.g. β-lactams), binding irreversibly to the 30S bacterial ribosome causing mistranslation, by inducing codon misreading on delivery of the aminoacyl RNA, with consequent inhibition of proteins synthesis. The most common mechanism of resistance to aminoglycosides is by enzymatic modification of the drug, inhibiting its binding to the ribosomal target site, therefore not compromising the protein synthesis. Three main types of aminoglycoside-modifying enzyme are produced by *S. aureus*, the aminoglycoside-3´´-*O*-phosphoryltransferase III [*aph*(3´)-III], the aminoglycoside-4´-*O*-phosphoryltransferase I [*ant*(4´)-I], and the aminoglycoside-6´-N-acetyltransferase/2´´-*O*-phosphoryltransferase [*aac*(6´)/*aph*(2´´)] (Krause *et al.*, 2016).

1.3.5.3. Resistance to chloramphenicol

Chloramphenicol is a bacteriostatic agent which binds to the 50S ribosomal subunit inhibiting the transpeptidation step during protein synthesis (Jensen & Lyon, 2009). The common mechanism of resistance to chloramphenicol is the enzymatic inactivation of the antibiotic by acetylation via different types of chloramphenicol acetyltransferases (CATs). All CATs enzymes are of classical A type, with subtypes A-7, -8 and -9 carried on the plasmids pC221, pC223 and pC194, respectively (Schwarz *et al.*, 2004, Foster, 2017). Another mechanism of

resistance is mediated by the *cfr* gene that encodes for a rRNA methylase responsible for the methylation of the 23S rRNA (position A2503). Resistance is also mediated by drug efflux, in this case by FexA, an efflux protein that belongs to the major facilitator superfamily (Foster, 2017).

1.3.5.4. Resistance to trimethoprim-sulphametoxazole (co-trimoxazole)

These two antifolate antibiotics act synergistically by inhibiting two essential enzymes in the bacterial biosynthesis of folic acid: sulphametoxazole (SMZ) inhibits the dihydropteroate synthetase (DHPS), which catalyses the formation of dihydrofolate from para-aminobenzoic acid, while trimethoprim (TMP) inhibits the dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate. The resistance to SMZ is related to the aminoacid substitutions in the chromosomally encoded DHPS which prevent the drug from binding to the enzyme. Similarly, resistante to TMP occurs by aminoacid substitutions in the chromosomally encoded DHFR or by horizontal acquisition of genes encoding additional DHFR enzymes that are not susceptible to the inhibition, allowing bypass of the blockaded chromosomal DHFR (Foster, 2017).

1.3.5.5. Resistance to other antibiotics

Resistance to fluoroquinolones

Fluoroquinolones are potent inhibitors of DNA topoisomerases, in the case of *S. aureus*; DNA gyrase and topoisomerase IV, which are essential enzymes involved in key cellular processes including DNA replication, resulting in impaired DNA replication (at lower concentrations) and cell death (at lethal concentrations). These enzymes (DNA topoisomerase IV and gyrase) are formed by two units of GrlA and GrlB each or GyrA and GyrB, respectively. Resistance to fluoroquinolones can occur via a range of mechanisms; in which the most common is due to the spontaneous mutations in the *grlA/B* and *gyrA/B* genes that cause amino acid substitutions in subunits GrlA/B and GyrA/B, respectively; altering the target protein structure and subsequently the fluoroquinolone-binding affinity of the enzymes, leading to drug resistance. These mutations arise in specific regions of the target genes, known as the quinolone resistance-determining region (QRDR). In addition to the

spontaneous mutations in QRDR, chromosomally-encoded multidrug efflux pumps actively remove fluoroquinolones and other drugs from the bacterial cell. One of such efflux pump is NorA, belonging to the major facilitator superfamily (MFS), which is associated with reduced susceptibility to fluoroquinolones as a result of overexpression of its encoding gene, *norA*. Therefore, the jointly action of efflux pumps and mutations in QRDR contribute to cross-resistance to distinct fluoroquinolones (Costa et al., 2015; Costa *et al.*, 2011; Tanaka *et al.*, 2000).

Resistance to macrolides, lincosamides and streptogramines (MLS_B)

The antibiotics from the classes of macrolides, lincosamides, and streptogramins B affect the bacterial cell in the same way. The target sites are the nucleotides A2058 and A2059 located in the V region of the 23S rRNA domain of 50S ribosome and, rarely, nucleotide A752 located within domain II. The binding of the antibiotic to the target leads to inhibition of polypeptide chain elongation with consequent stoppage of protein synthesis (Miklasińska-Majdanik, 2021). The resistance to MLS_B can be mediated by three main mechanisms: (a) modification of the ribosomal binding site, (b) ribosome protection via ATP-binding-cassette family (ABC-F) proteins, and (c) enzymatic modification of antibiotics (Feßler *et al.*, 2018). The modification of the target site is mediated by the adenyl-N-methyltransferase erythromycin resistance methylase (Erm) enzymes encoded by *erm* genes located on plasmids and transposons. The Erm enzymes are responsible for the methylation of adenine, which results in the formation of N-methyl adenine or N, N-dimethyl adenine and consequently post-transcriptional modification of the 23S rRNA structure. Adenine methylation prevents macrolides from binding to their target site on the bacterial ribosome. Because MLS_B antibiotics share a common binding site, cross-resistance is thereby created. The *erm* genes, particularly *ermA*, *ermB*, and *ermC*, are the most important in the development of MLS_B resistance in *S. aureus* (Miklasińska-Majdanik, 2021). The *msr* genes determine a second mechanism of resistance, which is manifested by resistance to macrolides and streptogramin B (MS_B phenotype). Resistance associated with *msr* genes was initially associated with active efflux (Ojo *et al.*, 2006), yet recent reports indicate that they play role of protection of the ribosomal target site (Sharkey *et al.*, 2016). Some *S. aureus* strains are

able to enzymatically inactivate macrolides via the production of esterazes encoded by *empC*, *ereA* or *ereB* genes, a mechanism not considered clinically relevant (Fyfe *et al.*, 2016).

Resistance to tetracycline

Tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome, and consequently stop elongation of synthesizing proteins. The resistance to tetracyclines is mainly conferred by three different mechanisms that can coexist in the same isolate; the active efflux of the drug, the protection of the tetracycline target site (ribosome protection), and the enzymatic inactivation of the drug (Pantosti *et al.*, 2007). All these mechanisms are based on the acquisition of one or several tetracycline resistance determinants, which are widely distributed among the *S. aureus*. The efflux of tetracyclines is mediated by different membrane-associated proteins called Tet proteins encoded by the plasmid-born genes *tet*(K) and *tet*(L). The protein-based ribosomal protection mechanism is normally encoded by *tet*(M) or *tet*(O), which consists in interaction with the ribosome causing the tetracycline to dislodge from the ribosome, thus protecting the bacterial cell from tetracycline's inhibitory activity. The chemical inactivation of tetracycline is an oxygen-dependent flavin-monooxygenase enzyme encoded by the *tet*(X) gene (Nelson & Levy, 2011).

Resistance to vancomycin

Vancomycin has been considered the drug of choice for treatment of severe MRSA infections, and the last line of defense against Gram-positive cocci infection (Choo & Chambers, 2016). The antibiotic binds to the D-Ala-D-Ala C-terminus of late peptidoglycan precursors (*N*-acetylmuramic acid and *N*-acetylglucosamine peptide subunits) with high affinity, preventing its incorporation into the peptidoglycan matrix, hence inhibiting the cell wall synthesis (Loll & Axelsen, 2000). There are two mechanisms of decreased susceptibility/resistance to glycopeptides in *S. aureus*. The first one is associated with a thickened and poorly cross-linked cell wall, leading to decreased susceptibility to the antibiotic and the detection of the vancomycin intermediate-resistant *S. aureus* (VISA) strains. This mechanism is usually associated with single patients submitted to prolonged

treatments and involves global adaptation of bacteria to the conditions of that particular patient (Gardet & Tomasz, 2014; Pereira *et al.*, 2007). The second mechanism is associated with the acquisition of the *vanA* operon from *Enterococcus* species, that alters the peptidoglycan precursor from D-Ala-D-Al to D-Ala-D-Lac, leading to the vancomycin high-level resistance exhibited by vancomycin-resistant *S. aureus* (VRSA) strains (McGuinness *et al.*, 2017).

Resistance to linezolid

Linezolid is a useful antibiotic in treatment of resistant Gram-positive cocci and bacillar infections. It acts mainly by inhibiting bacterial protein synthesis, by binding to the central loop of domain V in the 23S fraction (P site) of the 50S ribosome and interfering with the formation of tertiary N-formylmethionine- tRNA- 70S initiation complex. Hence, it impairs the initiation of protein synthesis. The resistance to linezolid is associated with mutations in the 23srRNA subunit domain V region of ribosomes leading to alteration of peptidyltransferase center, where conserved regions of ribosome interact directly with linezolid (Mendes *et al.*, 2014). Additional resistance is mediated by plamids encoding the Cfr protein, a methyltransferase that methylates C-8 of A2503 in 23S rRNA preventing the drugs from binding (Foster, 2017).

Resistance to daptomycin

Daptomycin is active against a wide-range of Gram-positive bacteria, including most clinically relevant MDR, MRSA and VISA strains. This antibiotic exerts its bactericidal effect by altering the bacterial cell envelope homeostasis, interacting with phospholipids of the cell membrane, in a process that has not been fully elucidated. The bactericidal activity of daptomycin depends on the presence of ionized calcium, in which the positive charge of the daptomycin-calcium complex is thought to facilitate the insertion of the antibiotic into the bacterial cell membrane (Tran *et al.*, 2015). The mechanisms of resistance to daptomycin are still yet to be completely understood. A suggested phenomenon that mechanistically links all the pathways of resistance to daptomycin is the “repulsion” of the antibiotic molecule from the cell surface, which is generally associated with an overall change in the net charge

of the bacterial surface (towards a more positive cell membrane) (Tran *et al.*, 2015).

1.4. Molecular epidemiology of *S. aureus*

1.4.1. Molecular tools for *S. aureus* typing

Historically, early typing of *S. aureus* strains relied mostly on phenotypic strain characteristics, such as susceptibility to bacteriophages or antibiotics. With the advent of molecular technologies, a variety of methods were introduced for bacterial typing (David *et al.*, 2013; van Belkum *et al.*, 2007). For *S. aureus*, these include macrorestriction analysis by pulsed-field gel electrophoresis - PFGE (Chung *et al.*, 2000; David *et al.*, 2013), the multilocus sequence typing -MLST (Enright *et al.*, 2000), *S. aureus* protein A typing - *spa* typing (Mellmann *et al.*, 2008), the staphylococcal chromosomal cassette typing - SCC*mec* typing (Oliveira & Lencastre, 2002), and the whole genome sequencing (WGS); allowing outbreak investigations and other epidemiological studies.

1.4.1.1. Macrorestriction analysis by PFGE

The PFGE (pulsed-field gel electrophoresis) analysis was initially considered the "gold standard" method for genotyping of *S. aureus*, because of its excellent discriminatory power, especially well suited for analysis of the local short-term epidemiology (i.e. outbreak investigations), as this method can often differentiate isolates that have undergone minor genetic changes (Chung *et al.*, 2000; David *et al.*, 2013). The method consists on the isolation of intact large chromosomal DNA molecules by lysing bacterial cells embedded in an agarose plug, followed by DNA digestion (within the agarose plug) by a rare cutting restriction enzyme (usually *Sma*I for *S. aureus*), to produce up to about 20 high-molecular weight DNA fragments. The digested DNA fragments (10 – 800 kb) are then separated in an agarose gel by alternating the electric field between spatially distinct pairs of electrodes, facilitating megabase (Mb) size DNAs to reorient and migrate at different speeds through the gel pores towards the anode, in a size dependent manner (Sharma-Kuinkel *et al.*, 2014). The macrorestriction patterns are then normalized and analysed by specific computer programs. Despite the excellent discriminatory power of PFGE, the method is labourious and the data interpretation is hampered by the lack of both interlaboratory reproducibility and a common

nomenclature (Chung *et al.*, 2000). These limitations were overcomed by the development of sequence-based methods such as MLST and *spa* typing, which provided fast, unambiguous, and exportable typing data.

1.4.1.2. MLST

MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of ~ 450 bp internal fragments of four to seven relatively conserved, housekeeping genes that encode essential proteins (Enright *et al.*, 2000; Saunders & Holmes, 2014). The accumulation of nucleotide changes in housekeeping genes is a relatively slow process and the allelic profile of a bacterial isolate is sufficiently stable over time for the method to be ideal for global epidemiology (Enright & Spratt, 1999), providing insights into bacterial evolutionary and population biology. The MLST scheme developed and validated for *S. aureus* involves amplification by polymerase chain reaction (PCR) of the following genes: carbamate kinase (*arcC*); shikimate dehydrogenase (*aroE*); glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*); triosephosphate isomerase (*tpi*); and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright *et al.*, 2000). For each of the seven loci (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*), different sequence polymorphisms are assigned arbitrary allele numbers by a centralized, publically available database (https://pubmlst.org/), and the seven assigned alleles combined into an allelic profile (e.g. 46, 75, 49, 44, 13, 68, and 60), that defines an unique sequence type (ST) (e.g. ST152) (Enright & Spratt, 1999). The use of a common publically available database for allele and ST assignment assures comparability and exportability of typing data between laboratories. Sequences with a single nucleotide difference are considered distinct alleles, and no weighting is applied to reflect the number of nucleotide differences between alleles (Maiden *et al.*, 2013). As there are many alleles at each of the seven loci, isolates are highly unlikely to have identical allelic profiles by chance, and isolates with the same allelic profile can be assigned as members of the same clone (Enright *et al.*, 2000). The relatedness between STs is inferred by different algorithms (Maiden *et al.*, 2013) that group isolates into clonal complexes (CCs) based on the number of differences between allelic profiles. One of these algorithms is the goeBurst (Francisco *et al.*, 2009), which assumes that a given genotype

increases in frequency in the population, as a consequence of a fitness advantage or of random genetic drift, becoming a founder clone in the population, and this increase is accompanied by a gradual diversification of that genotype, by mutation accumulations and recombination events, forming a cluster of phylogenetically closely related strains. As represented in Figure 1.3, this diversification is reflected in the appearance of STs differing only in one housekeeping gene sequence from the founder genotype - single locus variants (SLVs). Subsequent diversification of those SLVs will result in the appearance of variations of the original genotype with more than one difference in the allelic profile: double locus variants (DLVs), triple locus variants (TLVs), and so on. Therefore, the application of the algorithm to an entire data set, results in a disjoint set of trees with a hypothetical pattern of descent for the analyzed strains showing the possible phylogenetic relatedness between STs. Those STs that match the central genotype (ST) at four or more loci, unless they more closely match another central genotype, are defined as belonging to the same CC (Jolley *et al.*, 2018), as shown in Figure 1.3.

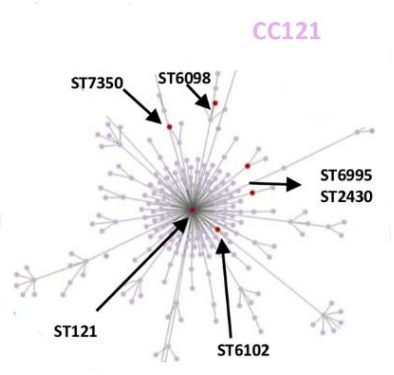

Figure 1.3. A goeBurst diagram exemplified for *S. aureus* CC121. The primary founder genotype (ST121) has three single-locus variants (ST2430, ST6102, ST6995), one double-locus variant (ST7350) and one triple-locus variant (ST6098). All the depicted STs belong to CC121.

Although MLST has become the "gold standard" method for population analysis, it is expensive and labor intensive, for its application to outbreak investigations and routine surveillance.

1.4.1.3. *Spa* typing

The *spa* typing is based on the sequencing analysis of the *spa* gene, which encodes protein A, a cell-wall component of *S. aureus* which interacts with immunoglobulins inhibiting phagocytosis (Hallin *et al.*, 2009). The *spa* gene is approximately 2,150 bp in length and encodes three regions: the Fc-binding region, the X region, and the C-terminal region. The X region (repeat region) consist of variable-number tandem repeats (VNTR). These contain 2 to 15 repetitive sequences consisting of 21 to 27 bp, which are polymorphic and diverse due to deletions and duplications of the repeats and occasionally due to point mutations (Lakhundi & Zhang, 2018). The *spa* types can be defined based in two standardized international nomenclatures defined by systems that follow a similar approach for *spa* types classification (Harmsen *et al.*, 2003; Koreen *et al.*, 2004). Each polymorphism is assigned a numerical (Ridom nomenclature) or alphanumerical code (eGenomics), in which the *spa* types are deduced from the order of specific repeats, as shown in Figure 1.4. The comparison between the two nomenclatures is easy and performed via computerized tools; therefore, the strain can be assigned the same *spa* type regardless of the nomenclature scheme used (Lakhundi & Zhang, 2018). The most common software used for *spa* types data analysis is the Ridom Staph type linked to a database system that enables straightforward semi-automated sequence analysis and type assignment by synchronizing laboratory typing data to a central *spa* server at www.SpaServer.ridom.de. As *spa* typing involves the interrogation of a single polymorphic locus, it is considered a suitable method for both local and global epidemiological studies (Hallin *et al.*, 2009; Koreen *et al.*, 2004). However, the approach of this method based on a single-locus typing can misclassify strains or lineages due to sequencing errors or recombination (Sabat *et al.*, 2013).

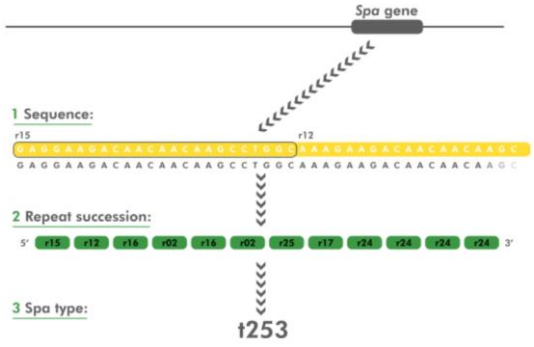

Figure 1.4. Schematic map of *spa* types assignment (Reproduced from https://www.applied-maths.com/applications/staphylococcus-aureus-spa-typing under permission from Biomérieux). Each new base composition of the polymorphic repeat found in a strain is assigned a unique repeat code. The repeat succession for a given strain determines its *spa* type.

Due to its simplicity, in addition of being less expensive and time-consuming (Koreen *et al.*, 2004) and agreement with MLST results, this single-locus marker (*spa* typing) is a useful tool for the epidemiology of *S. aureus.*

1.4.1.4. SCC*mec* typing

The staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element that carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mecA* and *mecC* genes in *S. aureus*. Therefore, the emergence of methicillin-resistant staphylococcal lineages is due to the acquisition and insertion of the SCC*mec* element into the chromosome of methicillin-susceptible strains, playing a role not only in antimicrobial resistance, but also in the molecular epidemiology and evolution of MRSA (Silva *et al.*, 2020). As described above (section 1.3.5.1), the different *S. aureus* SCC*mec* elements share a similar backbone structure, consisting on (i) *mec* complex, composed of *mec* operon; (ii) *ccr* gene complex, composed of cassette chromosome recombinase (*ccr*) gene(s), and (iii) the joining regions (J regions) (IWG-SCC, 2009).

Distinct multiplex PCRs have been widely used to detect multiple components in SCC*mec* to identify the SCC*mec* types and subtypes (Kondo *et al.*, 2007; Milheirico *et al.*, 2007; Milheiriço *et al.*, 2007; K. Zhang *et al.*, 2005, 2012). The SCC*mec* elements are defined into types by the combination of the *mec* gene complex and *ccr* gene complex (Table 1.2); the two key elements of the cassette respectively responsible for beta-lactam resistance

phenotype, integration and excision of SCC*mec*. Currently, there are fourteen *S. aureus* SCC*mec* types described (Table 1.2). From these, SCC*mec* types I, II and III present as large elements harbouring genes that confer resistance to several antibiotic classes and are primarily found in HA-MRSA; while the SCC*mec* types IV and V correspond to smaller elements found both in HA-MRSA and CA-MRSA. The SCC*mec* elements are further divided into subtypes centered on the variations in the J regions within the same combination of *mec*-*ccr* complex. All *S. aureus* SCC*mec* contain *mecA*, except the type XI which contains the homologue *mecC*; *mecA* and *mecC* encode for PBP2a and PBP2c, respectively, a peptidoglycan transpeptidase with low affinity for most β-lactam antibiotics as described above (section 1.3.5.1 - Resistance to ß-lactams) (Lakhundi & Zhang, 2018).

Table 1.2. Types of *S. aureus* SCC*mec* (Adapted from IWG-SCC, 2022).

***SCCmec* types**	***mec* gene complex**	***ccr* gene complex**	***mec* determinant**
I	B	1 (A1B1)	*mecA*
II	A	2 (A2B2)	*mecA*
III	A	3 (A3B3)	*mecA*
IV	B	2 (A2B2)	*mecA*
V	C2	5 (C1)	*mecA*
VI	B	4 (A4B4)	*mecA*
VII	C1	5 (C1)	*mecA*
VIII	A	4 (A4B4)	*mecA*
IX	C2	1(A1B1)	*mecA*
X	C1	7 (A1B6)	*mecA*
XI	E	8 (A1B3)	*mecC*
XII	C2	9 (C2)	*mecA*
XIII	A	9 (C2)	*mecA*
XIV	A	5 (C1)	*mecA*

SCC*mec* typing is used for classification of SCC*mec* element among MRSA strains (Oliveira & Lencastre, 2002). The combination of the MLST type, *spa* type and the SCC*mec* type, defined as the "clonal type" is now used for the international nomenclature of MRSA clones (Saunders & Holmes, 2014).

1.4.1.5. Whole genome sequencing (WGS)

The WGS is the most advanced approach for identification of genetic diversity in any organism. This method has the advantage of providing complete information on the genome of a strain, being an attractive tool for epidemiological purposes. The genetic relatedness among isolates by WGS can be determined throughout comparison of different genomes with single-nucleotide resolution (Hilt & Ferrieri, 2022). The WGS method surpass all the methods described above (PFGE, MLST, *spa* typing, and SCC*mec* typing), as the results from the genome assemblies can be inferred with high rate of accuracy (Quainoo *et al.*, 2017; Rodriguez *et al.*, 2015). However, the main challenges in using WGS for molecular typing is the availability of significant computer resources (i.e. web-developed bioinformatics software pipelines) for rapid processing and data analysis and the timely availability of well-trained bioinformaticians (Quainoo *et al.*, 2017), as well as quality control issues (Rossen *et al.*, 2018).

1.4.2. Global and regional molecular epidemiology of *S. aureus* and SAB-related *S. aureus*

The molecular epidemiology of *S. aureus* presents variation depending on the geographic location, disease burden and risk factors for the infection. The *S. aureus* ST5, ST8, ST22, ST30 and ST45 are the lineages frequently worldwide reported, while ST612 has been sporadically described in specific regions in South Africa, Tanzania and Australia (Table 1.3) (Lakhundi & Zhang, 2018; Moremi *et al.*, 2016). The available reports for African countries, describe a heterogeneous distribution of the major African methicillin-susceptible *S. aureus* clones (e.g. ST5, ST8 and ST15) and predominance of ST88-MRSA-III/IV, also known as the "African clone" (Table 1.3). However, detailed epidemiological and molecular characterization of *S. aureus* infection in this continent (Lawal *et al.*, 2022, Schaumburg *et al.*, 2014) are still scarce compared to the ones available from high-income countries, reinforcing the need of additional studies to understand the trend of epidemiology of this important pathogen in Africa. Data from Mozambique's bordering countries revealed distinct epidemiological features of *S. aureus*, with Tanzania presenting an abrupt increase of MRSA in less than 10 years with predominance of the "African clone" ST88 (Mzee *et al.*, 2021),

while the ST5, ST22, ST30, ST36, ST239 and ST612 predominated among MRSA strains circulating in South Africa (Abdulgader *et al.*, 2020; Oosthuysen *et al.*, 2014; Perovic *et al.*, 2015, 2017). A study from Democratic Republic of the Congo (Central Africa) revealed predominance of t1476-ST8-SCC*mec*V and ST152 (t355/t1096/t5691) among patients with bacteraemia infected by MRSA and MSSA, respectively (Vandendriessche *et al.*, 2017). Another review reported predominance of t037 and t064 mainly among MRSA strains, and the t084 mainly among MSSA strains in African countries (Asadollahi *et al.*, 2018).

A recent systematic review analyzed the dynamics of clonal distribution of MRSA for about one third of the African countries (16 out of 54) in the last six years (November 2014 – December 2020) (Lawal *et al.*, 2022); data for the remaining 38 countries, including Mozambique, were not described due to scarcity of information. The study reported high genetic heterogeneity among MRSA, with the CC5, CC8, and CC88 being widely distributed across the continent. In addition, it was observed an increase in the number of MRSA clones circulating in the period of analysis (November 2014 - December 2020) (Lawal *et al.*, 2022), compared to similar analysis from a previous period (before 31 October 2014) (Abdulgader *et al.*, 2015). The CC1-MRSA, which was previously described only in Nigeria and Tunisia (Abdulgader *et al.*, 2015), was recently reported in Egypt, Morocco, São Tomé and Príncipe, South Africa, and Uganda (Lawal *et al.*, 2022) and was absent in Tunisia. Similarly, the ST22/CC2-SCC*mec*IV[2B], previously documented only in South Africa (Abdulgader *et al.*, 2015), has now been also described in Angola, Algeria, Egypt, Kenya, and Morocco (Lawal *et al.*, 2022). Also, the CC152-MRSA, primarily reported only in Nigeria (Abdulgader *et al.*, 2015), has been recently identified in the Democratic Republic of the Congo, Kenya, and South Africa (Lawal *et al.*, 2022). Contrary, clone ST239/ST241, previously described as a major clone on the continent, declined significantly being found only in Egypt, Ghana, Kenya, and South Africa (Lawal *et al.*, 2022). Regardless of this scenario, some MRSA clones were still limited to specific countries and regions, as the ST80/CC80-SCC*mec*IV[2B] and ST612/CC8-SCC*mec*IV[2B], which were identified only in North African countries and in South Africa, respectively (Lawal *et al.*, 2022).

Table 1.3. Main *S. aureus* clones circulating in distinct geographic locations.

ST	Geographic location (continent/region)	References
ST5	Africa, Australia, Asia, Europe, and North America	(Effelsberg *et al.*, 2020; Lakhundi & Zhang, 2018)
ST8		
ST22		
ST30		
ST45		
ST239-III	Southeast Asia and Africa	(Mohamad *et al.*, 2022)
ST22-IV		
ST241-III		
USA300-LV	Southern America	(Junie *et al.*, 2018; Lee *et al.*, 2018)
USA1100		
USA300	North America	
ST80-IV	Europe	
ST93-IV	Australia	
ST88-IV	Africa	(Lawal *et al.*, 2022)
ST5		
ST22-IV		
ST152		
ST8		

1.4.3. Molecular epidemiology of SAB-related *S. aureus* in Mozambique

Previous studies on molecular characterization of *S. aureus* in our setting focused on isolates from skin and soft tissue infections (Ceccarelli *et al.*, 2005; Ruffing *et al.*, 2017; Van der Meeren *et al.*, 2014b) or only analyzed a small fraction of bacteraemic *S. aureus* isolates (Vubil *et al.*, 2017). The molecular characterization of a subset of *S. aureus* causing paediatric bacteraemia isolated over a 9-years period showed high genetic diversity, with predominance of clones CC5, CC8, CC15 and CC25 (Vubil *et al.*, 2017). Although previous reports provided a snapshot on molecular characterization of *S. aureus* causing infections in Mozambique, they were limited by the small number (and possible bias on sample selection) of

the isolates analysed and short period of analysis (2001-2009), which did not allow to draw robust conclusions on the potential impact of circulating strains on clinical outcome. Therefore, solid data on clinical and molecular epidemiology is critical to call awareness of the importance on this pathogen in Mozambique and may support the need to better characterize this pathogen throughout the country. Hereby, we expanded the previous studies to investigate the epidemiology of SAB and to perform the characteriazation of SAB-related *S. aureus* isolated over two decades (2001 - 2019) within the invasive bacterial disease surveillance among children aged < 5 years admitted to the Manhiça District Hospital, Southern Mozambique.

1.5. Objectives

The general objective of this PhD research project is to describe the epidemiology and clinical presentation of *Staphylococcus aureus* bacteraemia, as well as the antibiotic susceptibility, its determinants and clonality of *S. aureus* causing bacteraemia in children less than 5 years of age admitted at the Manhiça District Hospital (MDH, Mozambique) over two decades (2001 - 2019). For this purpose, the following specific objectives were set:

- ✓ To describe the burden and epidemiological trend of SAB in children <5 years admitted at the MDH.
- ✓ To describe the clinical data of children with *S. aureus* bacteraemia *versus* other bacteraemia.
- ✓ To ascertain and compare the phenotype of not susceptibility to antimicrobials of SAB related-*S. aureus* strains, in particular MDR/MRSA *versus* non-MDR/MSSA.
- ✓ To assess and compare the impact of SAB (duration of hospitalization and associated mortality) among patients infected with MDR/MRSA *versus* non-MDR/MSSA.
- ✓ To determine the clonality of *S. aureus* circulating in the Manhiça district by molecular typing.
- ✓ To assay the virulence potential of representative *S. aureus* strains by screening PVL determinants, biofilm production and performing virulence assays in the infection model of *Galleria mellonella*.

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CHAPTER 2 – Epidemiology and clinical presentation of community-acquired *Staphylococcus aureus* bacteraemia in children under 5 years of age admitted to the Manhiça District Hospital, Mozambique, 2001-2019

This chapter is based on the research article:

Garrine M, Quintó L, Costa SS, Messa A, Massinga AJ, Vubil D, Nhampossa T, Massora S, Acácio S, Cossa A, Sigaúque B, Bassat Q, Couto I, Mandomando I (2023). Epidemiology and clinical presentation of community-acquired *Staphylococcus aureus* bacteraemia in children under 5 years of age admitted to the Manhiça District Hospital, Mozambique, 2001–2019. *Eur J Clin Microbiol Infect Dis*. 42(5):653–9. doi: 10.1007/s10096-023-04580-2.

Author´s contribution to this manuscript

M. Garrine participated in the study conceptualization, data curation, investigation, methodology, formal analysis, software, visualization, validation, original draft writing, writing-review and editing.

2.1. Abstract

Staphylococcus aureus bacteraemia (SAB) is one of the most common bloodstream infectionhs globally. Data on the burden and epidemiology of community-acquired SAB in low-income countries are scarce but needed to define preventive and management strategies. Blood samples were collected from children <5 years of age with fever or severe disease admitted to the Manhiça District Hospital for bacterial isolation, including *S. aureus*. Between 2001 and 2019, 7.6% (3,197/41,891) of children had bacteraemia, of which 12.3% corresponded to SAB. The overall incidence of SAB was 56.1 episodes/100,000 children-years at risk (CYAR), being highest among neonates (589.8 episodes/100,000 CYAR). SAB declined significantly between 2001 and 2019 (322.1 to 12.5 episodes/100,000 CYAR). In-hospital mortality by SAB was 9.3% (31/332), and significantly associated with infections by multidrug-resistant (MDR) strains (14.7%, 11/75 *vs.* 6.9%, 14/204 among non-MDR, $p = 0.043$) and methicillin-resistant *S. aureus* (33.3%, 5/15 *vs.* 7.6%, 20/264 among methicillin-susceptible *S. aureus*, $p = 0.006$). Despite the declining rates of SAB, this disease remains an important cause of death among children admitted to MDH, possibly in relation to the resistance to the first line of empirical treatment in use in our setting, suggesting an urgent need to review current policy recommendations.

Keywords: Bacteraemia, epidemiology, incidence, paediatric, *Staphylococcus aureus*.

2.2. Background

Staphylococcus aureus bacteraemia (SAB) is one of the most common bloodstream infections worldwide (Bai *et al.*, 2022; Kern and Rieg, 2020). In 2019, *S. aureus* was amongst the top three pathogens responsible for global deaths associated with antimicrobial resistance (AMR), with methicillin-resistant *S. aureus* (MRSA) causing more than 100,000 annual deaths (Murray *et al.*, 2022). While SAB epidemiology is well described in high-income countries (Bai *et al.*, 2022; Hassoun *et al.*, 2017), data from low-income countries, particularly in sub-Saharan Africa, remain scarce (Marchello *et al.*, 2019; Chukwumeze *et al.*, 2021). This study aims to describe the burden, epidemiological trends and clinical presentation of community-acquired SAB among children aged <5 years in southern Mozambique, between 2001 and 2019.

2.3. Material and methods

The study was conducted at the Manhiça District Hospital (MDH) by the *Centro de Investigação em Saúde de Manhiça* (CISM). The Manhiça district, a rural area in southern Mozambique, has a subtropical climate, with a warm and rainy season (November to April) followed by a cool and drier season (Sacoor *et al.*, 2013). The geographical and socio-demographic characteristics of the study community are described elsewhere (Sacoor *et al.*, 2013; Nhacolo *et al.*, 2021). CISM's health and demographic surveillance system (HDSS) documents vital events and migrations in the Manhiça district (2,380 km^2) with an estimated population of 201,845 inhabitants (27,560 children aged <5 years) living in 46,441 households (Nhacolo *et al.*, 2021). Since 1997, the CISM and MDH operate a 24-hour morbidity surveillance, which includes the collection of clinical data of all paediatric patients and a systematic collection of a single venous blood sample for bacterial isolation upon hospital admission for all children aged < 2 years, and for children aged 2 and < 15 years with axillary temperature ≥ 39 ºC or with signs of severe illness (Sigaúque *et al.*, 2009). All the data analysed were obtained from the microbiological and morbidity surveillance databases of the study area (Sacoor *et al.*, 2013; Nhacolo *et al.*, 2021; Sigaúque *et al.*, 2009). Minimum community-based incidence rates of SAB and 95% CIs were calculated considering individual time at risk for children residing in the CISM study area excluding

periods of migration. In calculating person-time, individuals were excluded during a lag period of 15 days after each episode of community-acquired bacteraemia. Negative binomial regression models were estimated to compare incidence rates. Score test for trend of rates with calendar year was assessed by Mantel-Haenszel type method. Logistic regression models were used to compare the prevalence of SAB among all admitted children. Wilcoxon rank sum tests were used for nonparametric comparisons. Nutritional status was assessed using weight-for-age z scores, calculated using the LMS method and the 2000 CDC growth reference charts (Ogden *et al.*, 2002). In-hospital case fatality rate (CFR) due to SAB was calculated for children with known outcome, excluding patients that left the hospital without medical permission (absconders) or those transferred to Maputo Central Hospital (MCH). Data on antibiotic resistance - MRSA and multidrug-resistant (MDR) phenotypes of this *S. aureus* collection (Garrine, M *et al.*, 2023b) were compared with clinical and epidemiological features using χ^2 or Fisher's exact test, considering a significance level of 5% (STATA version 17, StataCorp LP, Texas, USA).

2.4. Results

2.4.1. Study population and positivity of SAB

From January 1, 2001, to December 31, 2019; 50,293 children aged <5 years were admitted to MDH, and blood cultures collected on admission for 83.3% (41,891) of these patients. Bacteraemia was diagnosed in 7.6% of cases (3,197/41,891) with *S. aureus* isolated in 0.9% (394/41,891) of the blood cultures, corresponding to 12.3% (394/3,197) of bacteraemic patients. The proportion of SAB was highest among children aged ≤28 days (31.4%, 120/382), followed by 24-59 months (10.3%, 64/620) and 29 days-11 months (10.2%, 113/1,112), and 12-23 months (8.9%, 97/1,083) ($p < 0.0001$). The rate of *S. aureus* isolation was similar among males and females (53.3%, 210/394 *vs.* 46.7%, 184/394, respectively).

2.4.2. Minimum community-based incidence rates

Of the 394 SAB episodes, only 39.6% occurred in children living within the HDSS area, yielding an overall minimum community-based incidence of 56.1 episodes (95% CI, 47.9-65.6) per 100,000 children-years at risk (CYAR). The highest incidence was observed among

children aged ≤28 days with 589.8 episodes (95% CI, 404.5-860.1) per 100,000 CYAR) and decreased with age ($p < 0.0001$): 92.5 episodes (95% CI, 68.8 - 124.3) for children aged 29 days - 11 months, 89.3 (95% CI, 68.4 - 116.6) for 12-23 months, and 18.7 episodes (95% CI, 13.2 - 26.6) per 100,000 CYAR aged 24-59 months. The over-time incidence trend analysis showed a decline throughout the years, from 322.1 (95% CI, 210.0 - 494.1) to 12.5 (95% CI, 3.1 - 49.8) episodes per 100,000 CYAR between 2001 and 2019 ($p < 0.0001$), Figure 2.1. The incidence rate was twice as high in the rainy season compared to dry season (77.3 [95% CI, 63.9 - 93.5] *vs.* 35.7 [95% CI, 27.1 - 47.2] episodes/100,000 CYAR, respectively; $p < 0.0001$).

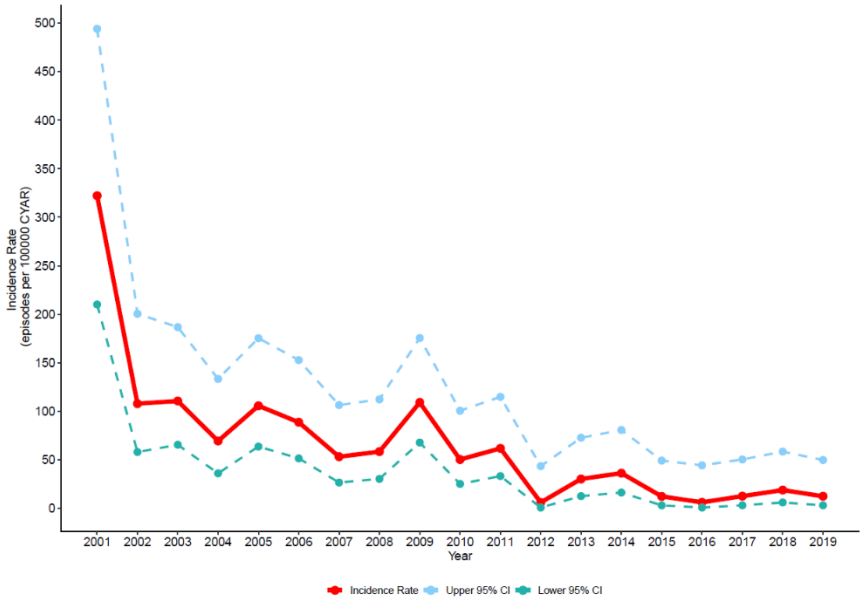

Figure 2.1. Trends of minimum estimates of community incidence rates of bacteraemic by *S. aureus* between 2001 and 2019. CYAR - Children-years at risk

2.4.3. Clinical data

Complete clinical records were only available for 66.7% (263/394) of SAB patients and 72.9% (2,044/2,803) of patients with other bacteraemia (by other bacterial pathogens). Patients with SAB were significantly more likely to have clinical malaria and less likely to have non-severe and severe anaemia, fever, clinical pneumonia, and dehydration, than patients with other bacteraemia (Table 2.1). The measure of Lambaréné Organ Dysfunction

Score (LODS) for illness severity was similar among patients with SAB and with other bacteraemia (Table 2.1), while the median length of stay (LOS) was higher in the second group (4 days [IQR: 3-7] *vs.* 5 days [IQR: 3-9], $p < 0.0001$; respectively).

Table 2.1. Clinical data displayed by children with *S. aureus* bacteraemia and with other bacteraemia, Manhiça District, Mozambique, 2001 – 2019

Clinical data		Other bacteraemia N=2,044 (%)	*S. aureus* bacteraemia N=263 (%)	Multivariate analysis OR[a] (95% CI[b])	*p*-value[c]
Age (months)[d]		14.9 (12.7)	11.0 (12.4)	0.9 (0.9 - 0.9)[e]	< 0.0001
Clinical Malaria		686 (34)	96 (37)	1.5 (1.1 - 2.1)	0.0123
Severe Malnutrition (WAZ < -3)[f]		491 (24)	40 (15)	0.8 (0.5 - 1.1)	0.1554
Anaemia	No-Anaemia	499 (24)	102 (39)	Reference	0.0008
	Non-Severe Anaemia	1283 (63)	136 (52)	0.6 (0.4 - 0.8)	
	Severe Anaemia	262 (13)	25 (10)	0.5 (0.3 - 0.8)	
Fever		1998 (98)	248 (94)	0.4 (0.2 - 0.9)	0.0269
Diarrhoea		548 (27)	60 (23)	1.1 (0.8 - 1.6)	0.6085
Vomiting		535 (26)	52 (20)	0.8 (0.6 - 1.2)	0.4508
Clinical Pneumonia		1180 (58)	93 (35)	0.6 (0.4 - 0.8)	0.0068
Splenomegaly		547 (27)	53 (20)	0.8 (0.6 - 1.1)	0.1446
Dehydration		437 (21)	30 (11)	0.4 (0.3 - 0.7)	0.0003
LODS[g]	0	1416 (69)	206 (78)	Reference	0.1275
	1	517 (25)	50 (19)	0.7 (0.5 - 1.0)	
	2	99 (5)	6 (2)	0.4 (0.2 - 1.1)	
	3	12 (1)	1 (0)	0.8 (0.1 - 6.8)	

[a]OR, odds ratio; [b]CI, confidence interval; [c]*p-value* determined by Logistic regression; [d]Arithmetic Mean (SD); [e]determined per month increase; [f]WAZ, weight-for-age z score, [g]Lambaréné Organ Dysfunction Score. Non-severe anaemia is a packed cell volume (PCV) in the range ≥15% - <33%, and severe anaemia as a PCV <15%. Acute clinical malaria was defined as a child admitted with a clinical diagnosis of malaria with confirmed *P. falciparum* asexual parasitaemia > 0 parasites/μL.

2.4.4. In-hospital case fatality rates

Of the 394 SAB patients, 32 were transferred to MCH, 29 left MDH without medical permission, and one had no medical record. The overall CFR for *S. aureus* was 9.3% (31/332), with the highest CFR in children aged 24-59 months (20.8%, 10/48), followed by 12-24 months (9.6%, 8/83), 29 days-11 months (9.3%, 9/97), and ≤ 28 days (3.8%, 4/104) (p = 0.021). We did not observe significant differences by calendar year (p = 0.957; data not shown); or between rainy and dry season (9.0%, 21/232 *vs.* 10.0%, 10/100, respectively; p = 0.785). Most deaths occurred early on admission, being documented within the first (14/31, 45.2%), second (4/31, 12.9%), third or beyond day of admission (13/31, 41.9%). Three patients died on the 15 days follow up: upon being discharged (n=1), transferred (n=1) or absconded from the hospital (n=1).

2.4.5. Co-infections among patients with SAB

One hundred and six (26.9%, 106/394) SAB patients had malaria, while 3.5% (14/394) presented co-infections with other bacteria, including streptococci and Gram-negative pathogens, and three were simultaneously infected by two *S. aureus* with distinct antibiotic resistance profiles. Data of 332 children with complete medical record were analysed to compare mortality of patients with SAB+malaria to those with SAB only (9.7%, 9/93 *vs.* 9.2%, 22/239, respectively; p = 1.000), and among patients with SAB co-infected by other bacterial pathogens to those with SAB only (33.3%, 4/12 *vs.* 8.4%, 27/320, respectively; p = 0.018). The median LOS was significantly extended among patients with SAB only compared to those with SAB+malaria (5 days [IQR: 3-8] *vs.* 3 days [IQR: 2-5], respectively; p < 0.001), and similar between patients with SAB only and those with SAB co-infected by other bacteria (4 days [IQR: 3-7] *vs.* 4 days [IQR: 2-11], respectively; p = 0.926).

2.4.6. Antibiotic therapy prescription

A complete record of antibiotic prescription was available for 287 (287/394, 72.8%) SAB patients, 91.6% (263/287) of which received antibiotics upon admission. Children treated with antibiotics were likely to present extended LOS than those not treated (5 days [IQR: 3-7] *vs.* 3 days [IQR: 2-4], respectively), although this difference was not significant (p =

0.163); while the mortality rate was similar (11.5%, 25/217 *vs.* 9.1%, 2/22, respectively; p = 1.000). Data on antibiotic prescribed were available since 2003 for 261 patients. The most administered antibiotics were gentamicin (63.9%, 167/261), ampicillin/amoxicillin (55.9%), ceftriaxone (24.5%), penicillin (21.8%), chloramphenicol (19.2%), co-trimoxazole (14.6%), and erythromycin (6.1%). The antibiotic prescription trend varied over the two decades (Figure 2.2). Ceftriaxone prescription increased throughout the years, while erythromycin and chloramphenicol prescription decreased. Use of ampicillin/amoxicillin and gentamicin remained similar throughout the surveillance period, while penicillin was frequently prescribed until 2014, and prescription of co-trimoxazole oscillated throughout the study period.

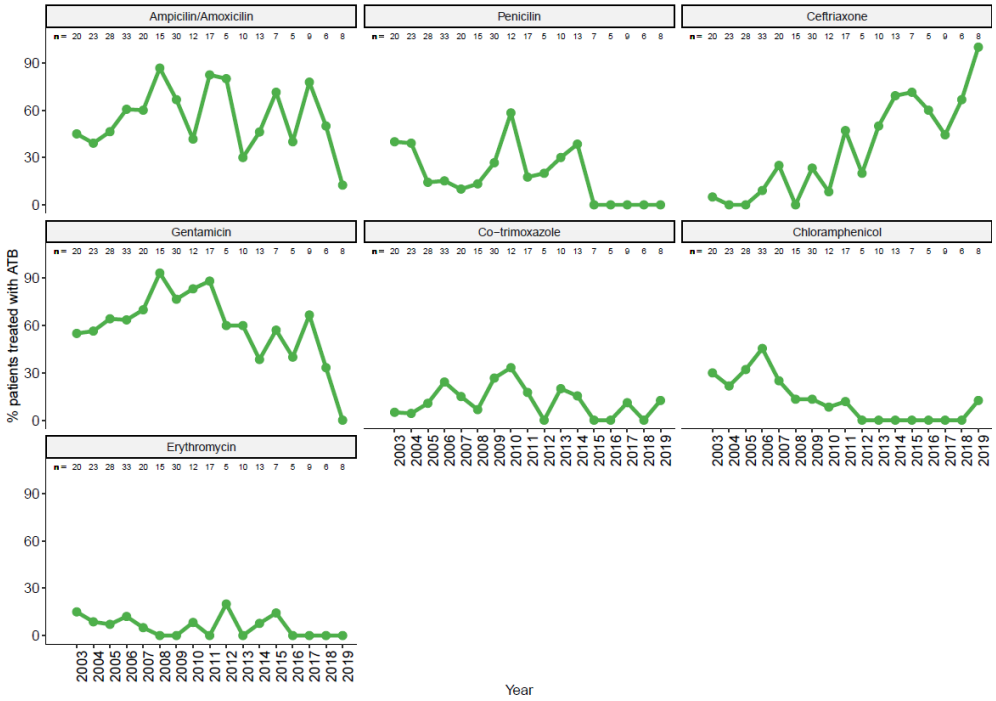

Figure 2.2. Trend of antibiotics prescribed among patients with bacteraemia by *S. aureus* admitted at the Manhiça District Hospital between 2003 and 2019. The number at the top (n) corresponds to the total of patients with bacteraemia by *S. aureus* admitted in that year

2.4.7. Comparison between AMR and outcome

Infections by MDR strains were likely to be associated with mortality compared to non-MDR strains (14.7%, 11/75 *vs.* 6.9%, 14/204, p = 0.043, respectively). Similarly, infections by

MRSA were associated with mortality compared to methicillin-susceptible *S. aureus* (MSSA) (33.3%, 5/15 *vs.* 7.6%, 20/264, respectively; $p = 0.006$). The median LOS was similar between patients infected by MDR and non-MDR strains (5 days, IQR: 3-8 *vs.* 4 days, IQR: 2-7, respectively; $p = 0.1629$), but extended among patients infected by MRSA strains compared to those infected by MSSA (7 days; IQR: 3-12.5 *vs.* 4 days, IQR: 3-7), respectively), although this difference was not significant ($p = 0.1345$).

2.5. Discussion

We described the epidemiological and clinical data of two decades of surveillance of community-acquired SAB among children admitted at MDH, showing a decline on SAB incidence in our setting. However, this incidence could be underestimated considering that only 39.6% of the cases were from the study area and identified through passive detection (severe cases for which health care was sought). This declining trend is similar to those observed for other invasive bacterial pathogens and clinical malaria previously reported in our setting (Sigaúque *et al.*, 2018; Mandomando *et al.*, 2015; Sigaúque *et al.*, 2013; Mandomando *et al.*, 2020). These downward trends probably reflect the improved socioeconomic and nutritional status, improved water supply, sanitary and overall hygiene conditions, more effective prevention of mother to child HIV transmission, and better access to antiretroviral treatment by patients infected by the HIV. The highest incidence of SAB detected among neonates was previously reported in our setting (Sigaúque *et al.*, 2009) and recently in Gambia (Odutola *et al.*, 2019), probably reflecting the relatively immature immune responses (Power Coombs *et al.*, 2013).

The SAB rate observed in our study (12.3%) was similar to the one previously reported in the same setting between 2001 and 2006 (Sigaúque *et al.*, 2009). However, this previous study did not evaluate the clinical characteristics and trend of bacteraemia by pathogen nor evaluated its association with malaria. In our study, SAB patients with co-infections by other bacteria were more likely to present a poor outcome calling for an early and accurate diagnosis for a proper inpatient clinical management. The rate of SAB plus co-infections with other bacteria (3.5%) and the CFR (7.8%) observed were similar to a previous report from South Africa (7% and 8.8%, respectively), a neighbouring country to Mozambique

(Naidoo *et al.*, 2013). However, the CFR reported here might be underestimated considering that more than 50% of paediatric deaths in the study area occur at home, with no previous hospital attendance (Sacarlal *et al.*, 2009). The high proportion of deaths reported early on admission suggests that the children sought hospital care in critical conditions, or that the infection was caused by isolates resistant to the initial empirical antibiotic therapy. We observed an association between mortality and infection by MRSA and MDR, in addition to the extended LOS among MRSA-infected patients. These findings corroborate previous reports showing persistent infection and high mortality among patients infected by MRSA compared to MSSA (Coombs *et al.*, 2016; Inagaki *et al.*, 2019), likely due to inappropriate empirical antibiotic therapy. Thus, identifying children with risk factors for MDR and MRSA infection will allow better guidance for empiric antibiotic therapy and a likely improved survival. In our study only a single blood culture was performed per patient, hindering the evaluation of infection persistence, although patients' clinical evolution was reassessed every day.

The Mozambican national guidelines recommend parenteral combination of penicillin plus gentamicin or chloramphenicol (less prescribed due to its toxicity, despite occasional use in case of antibiotic stock out) for children >2 years or ampicillin plus gentamicin as empirical antibiotic therapy in younger children or those cases of severe malnutrition. Treatment is reassessed based on clinical evolution and blood culture results using ceftriaxone for infections by MDR strains or severe disease. Our data demonstrated that these antibiotics were the most used in our setting, with ceftriaxone increasingly prescribed throughout the years. We recently reported low rates of SAB caused by MRSA or gentamicin-resistant strains (<6%), but high for penicillin-resistant strains (90%) in our setting (Garrine *et al.*, 2023). This resistance may challenge the empirical therapy based on combination of ampicillin/penicillin plus gentamicin, while ceftriaxone may continue as a valuable alternative. Due to the lack of complete information on treatment duration, number of doses of antibiotic given and prescription alterations, we were unable to assess the quality and appropriateness of antimicrobial therapy and relate it to patient's outcome.

2.6. Conclusion

In spite of the general declining rates of SAB in our population, this disease remains an important cause of death among children admitted to MDH, possibly in relation to the resistance to the first line of empirical treatment in use in our setting, suggesting an urgent need to review current policy recommendations.

2.7. Ethics approval

The morbidity surveillance described in this study is included in the ongoing morbidity surveillance system established as part of the CISM´s health and demographic surveillance system (HDSS) approved by the Institutional Bioethics Committee for Health at CISM, and from the National Bioethics Committee for Health.

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CHAPTER 3 - Antimicrobial resistance and clonality of *Staphylococcus aureus* causing bacteraemia in children admitted to the Manhiça District Hospital, Mozambique, over two decades

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Author´s contribution to this manuscript

M. Garrine participated in the study conceptualization, data curation, investigation, methodology, formal analysis, software, visualization, validation, original draft writing, writing-review and editing.

3.1. Abstract

3.1.1. Background

Staphylococcus aureus is one of the main causes of bacteraemia, associated with high mortality, mainly due to the occurrence of multidrug resistant (MDR) strains. Data on antibiotic susceptibility and genetic lineages of bacteraemic *S. aureus* are still scarce in Mozambique. The study aims to describe the antibiotic susceptibility and clonality *of S. aureus* isolated from blood cultures of children admitted to the Manhiça District Hospital over two decades (2001–2019).

3.1.2. Methods

A total of 336 *S. aureus* isolates detected in blood cultures of children aged <5 years were analyzed for antibiotic susceptibility by disk diffusion or minimal inhibitory concentration, and for the presence of resistance determinants by PCR. The clonality was evaluated by *Sma*I-PFGE, *spa* typing, and MLST. The SCC*mec* element was characterized by SCC*mec* typing.

3.1.3. Results

Most *S. aureus* (94%, 317/336) were resistant to at least one class of antibiotics, and one quarter (25%) showed a MDR phenotype. High rates of resistance were detected to penicillin (90%) and tetracycline (48%); followed by erythromycin/clindamycin (25%/23%), and co-trimoxazole (11%), while resistance to methicillin (MRSA strains) or gentamicin was less frequent (≤5%). The phenotypic resistance to distinct antibiotics correlated well with the corresponding resistance determinants (Cohen's κ test: 0.7–1.0). Molecular typing revealed highly diverse clones with predominance of CC5 (17%, 58/336) and CC8 (16%), followed by CC15 (11%) and CC1 (11%). The CC152, initially detected in 2001, re-emerged in 2010 and became predominant throughout the remaining surveillance period, while other CCs (CC1, CC5, CC8, CC15, CC25, CC80, and CC88) decreased over time. The 16 MRSA strains detected belonged to clones t064-ST612/CC8-SCC*mec*IVd (69%, 11/16), t008-ST8/CC8-SCC*mec*NT (25%, 4/16) and t5351-ST88/CC88-SCC*mec*IVa (6%, 1/16). Specific clonal lineages were associated with extended length of stay and high in-hospital mortality.

3.1.4. Conclusions

We document the circulation of diverse MDR *S. aureus* causing paediatric bacteraemia in Manhiça district, Mozambique, requiring a prompt recognition of *S. aureus* bacteraemia by drug resistant clones to allow more targeted clinical management of patients.

Keywords: Paediatric, bacteraemia, *Staphylococcus aureus*, MRSA, MDR, *spa* typing, MLST, Mozambique.

3.2. Introduction

Staphylococcus aureus bacteraemia (SAB) is one of the most common bloodstream infections worldwide (Kern and Rieg, 2020; Bai *et al.*, 2022). The mortality associated with SAB is higher (29%–63%) (Kaasch *et al.*, 2014) compared to bloodstream infections caused by other Gram-positive pathogens (Gijón *et al.*, 2016). The burden of SAB is increasing around the globe (Kern and Rieg, 2020) and the treatment of affected patients is challenged by the emergence of multidrug resistant (MDR) and methicillin-resistant *S. aureus* (MRSA) strains (Murray *et al.*, 2022). Most antibiotic resistance exhibited by *S. aureus* is due to resistance genes encoded on the chromosome or those acquired by horizontal transfer from other *S. aureus* strains, as well as from other bacteria (Vestergaard *et al.*, 2019). Furthermore, the global prevalence of MRSA is related to the dissemination of pandemic clones, and acquisition of the Staphylococcal chromosomal cassette *mec*—SCC*mec* element (which harbours the *mec* gene, encoding methicillin resistance) by local methicillin-susceptible *S. aureus* (MSSA) (Lee *et al.*, 2018). These reasons lead the World Health Organization (WHO) to list MRSA as priority target to guide research, discovery and development of new antibiotics, because of its ability to rapidly develop resistance against multiple antibiotic classes hence limiting therapeutic options (Tacconelli *et al.*, 2018).

Molecular typing studies on *S. aureus* have been using well-described methods, such as pulsed-field gel electrophoresis (PFGE) (Chung *et al.*, 2000), multilocus sequence typing (MLST) (Enright *et al.*, 2000), staphylococcal protein A typing (*spa* typing) (Frénay *et al.*, 1996) and SCC*mec* typing (Zhang *et al.*, 2005). These methods allow investigating outbreaks

and long-term epidemiological studies (Strommenger *et al.*, 2006; Mellmann *et al.*, 2008). More recently, the introduction of WGS tools has allowed exhaustive strain characterization (Raven *et al.*, 2019). However, detailed molecular characterization of clinical *S. aureus* from Africa has been largely neglected in the past (Schaumburg *et al.*, 2014). Although there are recent data regarding molecular characterization of *S. aureus* originating from different African countries (Perovic *et al.*, 2017; Ruffing *et al.*, 2017; Amoako *et al.*, 2019; Kyany'a *et al.*, 2019; Mzee *et al.*, 2021), they are still scarce compared to the ones available from other regions of the globe (Toleman *et al.*, 2017; Baig *et al.*, 2020; Cabrera *et al.*, 2020), reinforcing the need for additional studies to understand the local epidemiology of this important pathogen (Schaumburg *et al.*, 2014; Tong *et al.*, 2015). *S. aureus* was previously reported as the third leading cause of bacteraemia among children in Mozambique (Sigaúque *et al.*, 2009) and the first cause among newborns and infants under the age of 3 months (Sigaúque *et al.*, 2018); yet, in-depth characterization are still lacking. Early molecular characterization of a sub-set of approximately 20% of paediatric *S. aureus* causing bacteraemia in our study community, provided a snapshot on *S. aureus* bacteraemia in our region, showing high strain diversity with predominance of the clonal complexes (CC) CC5, CC8, CC15, and CC25, and the *spa* types t064 and t084 (Vubil *et al.*, 2017). However, one important study limitation was the small number of isolates analysed and possible bias on sample selection, which did not allow drawing robust conclusions on the potential contribution and role of MDR/MRSA impact on clinical outcome. We expanded this earlier study and found a declining rate of SAB, although the disease remains an important cause of child mortality in our study area (Taylor *et al.*, 2020; Garrine *et al.*, 2023), possibly in relation to the resistance to the first line of empirical treatment in use, suggesting an urgent need to review current policy recommendations (Garrine *et al.*, 2023). Therefore, we herein aim to fill the gap of knowledge associated with paediatric SAB, by describing the antibiotic susceptibility, presence of resistance determinants and clonality of *S. aureus* causing bacteraemia among children under 5 years of age in Manhiça district, Mozambique, in the last two decades (2001–2019).

3.3. Methodology

3.3.1. Site description

The Manhiça District Hospital (MDH) is a referral health facility for Manhiça district, a rural area located 80 km North of Maputo city, Southern Mozambique. A full description of the geographical and socio-demographic characteristics of the study community has been presented and updated elsewhere (Sacoor *et al.*, 2013; Nhacolo *et al.*, 2021). The *"Centro de Investigação em Saúde de Manhiça"* (CISM) has a continuous health and demographic surveillance system for vital events and migrations since 1996, currently covering the entire district with an estimated population of 201,845 inhabitants in 46,441 households (Nhacolo *et al.*, 2021).

3.3.2. Specimen collection and *S. aureus* isolation

Since 1997, the CISM and MDH have jointly operated a 24 h morbidity surveillance, with standardized collection of clinical data for all paediatric patients (<15 years of age) and a specific microbiological surveillance based on the systematic collection of blood cultures among all admitted patients (Sigaúque *et al.*, 2009). Specifically, as part of microbiological surveillance, a single venous blood sample (1–3 mL) for bacterial isolation is routinely collected upon hospital admission for all children aged <2 years, and for children aged between 2 and <15 years with axillary temperature ≥39°C or with signs of severe illness, as described elsewhere (Sigaúque *et al.*, 2009). In this study we focused our analysis on children aged <5 years, as 95% of *S. aureus* were isolated from this group.

3.3.3. Antimicrobial Susceptibility Testing

Three hundred and thirty-six frozen *S. aureus* isolates were retrieved and tested for antimicrobial susceptibility by Kirby–Bauer disk diffusion, or determination of minimal inhibitory concentration (MIC) by *E*-test. Results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). According to the CLSI guidelines, "not susceptibility" profiles included isolates categorized as intermediate or resistant (CLSI, 2023). Multidrug resistance was defined as not susceptibility to at least one agent in at least three unrelated classes of antibiotics (Magiorakos *et al.*, 2012). The isolates

were tested against the following antibiotics: cefoxitin (FOX, 30 μg), penicillin (PEN, 10 units), ciprofloxacin (CIP, 5 μg), chloramphenicol (CHL, 30 μg), erythromycin (ERY, 15 μg), gentamicin (GEN, 10 μg), tetracycline (TCY, 30 μg), trimethoprim/sulfamethoxazole "co-trimoxazole" (SXT, 1.25/23.75 μg), clindamycin (CLID, 2 μg), daptomycin (DAP, 0.016–256 μg/mL), linezolid (LNZ, 0.016–256 μg/mL) and vancomycin (VAN, 0.016–256 μg/mL) (Mast Group, Ltd., Merseyside, United Kingdom). The cefoxitin disk was used as a surrogate for oxacillin resistance, to screen putative MRSA. Inducible clindamycin resistance was detected by the *D*-test for all isolates resistant to erythromycin and susceptible or intermediate to clindamycin. *S. aureus* strains ATCC®25923™ and ATCC®29213™ were used as quality control for disk diffusion and E-test, respectively (CLSI, 2023).

3.3.4. Screening of resistance determinants

S. aureus showing a not susceptibility profile were screened for the presence of the corresponding resistance determinants by conventional PCR. Briefly, the isolates were cultivated into blood agar plates and incubated overnight at 37°C. Afterward, one colony was selected from the blood agar plate and inoculated into 5 mL of BD Tryptic Soy Broth (Becton-Dickinson, Heidelberg, Germany) followed by overnight incubation at 37°C. Upon incubation, crude DNA was extracted by the boiling method according to an established protocol (Alexopoulou *et al.*, 2006), and screened by PCR targeting the corresponding resistance genes of interest, namely, *blaZ*, *mecA*, *tet*(K), *tet*(M), *tet*(L), *erm*(C), *erm*(A), *msr*(A), *aacA-aphD*, *dfr*(G), *dfrA*(S1), and *cat*p_{C221}, using specific primers and conditions (Supplementary Table S3.1). The main resistance determinants encoding for resistance to penicillin (*blaZ*), cefoxitin (*mecA*) and tetracycline (*tet*(K)) were screened in the entire *S. aureus* collection. The amplification products were separated in 1.5% agarose gels stained with ethidium bromide, using the 1 Kb plus or 100 bp DNA ladder (Bio-Rad) as molecular size markers.

3.3.5. Screening of mutations in quinolone-resistance determining region (QRDR) of *grlA* and *gyrA* genes

The strains not susceptible to ciprofloxacin were screened for mutations in the quinolone-resistance determining region (QRDR) of *grlA* and *gyrA* genes, using specific primers and conditions (Supplementary Table S3.1). The amplicons were purified using the NZYGelpure kit (NZYTech, Lisbon, Portugal) and sequenced by the Sanger method at STAB-Vida (Caparica, Portugal). Sequence alignment analyses with appropriate reference sequences searched in the National Center for Biotechnology Information (NCBI, Bethesda, MD, United States) public repository database were conducted using MEGA 11 (Tamura *et al.*, 2021) to identify mutations associated with fluoroquinolone resistance (Hooper, 1999; Jones *et al.*, 2000).

3.3.6. Molecular typing and inference of CCs

Amplification and sequencing of the hypervariable region of the *spa* gene was carried out for the entire collection ($n = 336$) as described elsewhere (Harmsen *et al.*, 2003). The *spa* types were assigned using the Ridom Staph Type database (Ridom GmbH, Würzburg, Germany, version 2.2.5). MLST was performed for at least two representative *S. aureus* from each *spa* type ($n = 168$), using the scheme previously described (Enright *et al.*, 2000). Allelic profiles, sequence types (STs) and CCs were assigned using the MLST *S. aureus* database (https://pubmlst.org/). Selected *S. aureus* ($n = 160$) were analyzed by PFGE to solve discrepancies and/or to increase the discriminatory power of MLST/*spa* typing results. The isolates were compared for their genetic relatedness by *Sma*I macrorestriction, according to the protocols described elsewhere (Chung *et al.*, 2000). The restriction patterns were analyzed using BioNumerics version 7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) with Dice coefficient (1% and 0.5% of tolerance and optimization, respectively). Groups of isolates showing at least 80% of similarity were considered to share the same profile (pulsotype), and those with similarity ≥97% were considered the same subtype (Carriço *et al.*, 2005). PFGE patterns found in a single isolate were designated single pulsotypes. Previous studies have shown high concordance between groupings obtained by *spa* typing with the classifications obtained by MLST or PFGE (Strommenger *et al.*, 2006; Mellmann

et al., 2008). Therefore, for isolates with no CC assigned by PubMLST, we inferred the CCs based on the agreement between *spa* type, PFGE and STs/CCs and, when necessary, data obtained from the literature (Supplementary Table S3.2). The clonal relatedness was inferred with the PHYLOViZ online version (Francisco *et al.*, 2012), considering the STs/CCs found in this study and all STs/CCs described for *S. aureus* in the PubMLST database until December 2022.

3.3.7. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing

We performed a multiplex PCR for identification of SCC*mec* types (I, II, III, and V) and subtypes (IVa, IVb, IVc, and IVd) for the MRSA strains, using primers and conditions previously described (Zhang *et al.*, 2005).

3.3.8. Data analysis

The statistical analyses were performed using STATA version 14.1 (StataCorp LP, College Station, Texas, United States). Categorical variables were compared using the χ^2 or Fisher's exact test when appropriate, and a *p*-value of 0.05 or lower was considered statistically significant. Age groups were categorized as neonates (≤28 days), infants (29 days–11 months), toddlers (12–23 months), and young children (24–59 months) (Ministério da Saúde-Mozambique, 2011). The antimicrobial susceptibility data were analyzed through WHONET version 19.8.6 (World Health Organization, Geneva, Switzerland). The level of agreement between antibiotic susceptibility testing among selected antibiotics (penicillin, cefoxitin and tetracycline) and the resistance determinants (*blaZ*, *mecA* and *tet*(K)) was determined by Cohen's κ test using GraphPad Prism(https://www.graphpad.com/quickcalcs/kappa1/). The κ coefficient was interpreted as no agreement ($\kappa < 0$), slight agreement (κ: 0.00–0.20), fair agreement (κ: 0.21–0.40), moderate agreement (κ: 0.41–0.60), substantial agreement (κ: 0.61–0.80), and almost perfect agreement (κ: 0.81–1.00) (Landis and Koch, 1977). The genetic diversity of the collection was calculated, based on the *spa* types and MLST, by Simpson's index of diversity (SID) with 95% confidence interval (http://darwin.phyloviz.net/).

3.4. Results

3.4.1. Demographic characteristics

From January 01, 2001 to December 31, 2019; 50,293 children aged <5 years were admitted to the MDH, and blood cultures were collected on admission for 83% (41,891) of the patients. Bacteraemia was diagnosed in 7.6% of cases (3,197/41,891) with *S. aureus* isolated in 0.9% (394/41,891) of the blood cultures, corresponding to 12.3% (394/3,197) of bacteraemic patients. The epidemiological and clinical characteristics of these patients have been described in a separate study, including the proportion of SAB as a cause of community bacteraemia across the several age strata (Garrine *et al.*, 2023). In that early study, the SAB incidence ranged from 322.1 to 12.5 episodes/100,000 children years at risk between 2001 and 2019 (Garrine *et al.*, 2023). The present work describes the analysis of the 336 *S. aureus* isolates recovered from 333 children with SAB over this period, including three children presenting two morphologically distinct isolates in the same blood culture.

3.4.2. Antimicrobial resistance profile

Overall, 94% (317/336) of the *S. aureus* tested were not susceptible (resistant or intermediate phenotype) to at least one antibiotic (Table 3.1). More specifically, 37% (124/336) were not susceptible to one antibiotic class, 32% (108/336) to two classes and 25% (85/336) were MDR. High frequencies of resistance were observed for penicillin (90%), tetracycline (48%) and erythromycin/clindamycin (25% and 23%, respectively), while resistance to chloramphenicol or gentamicin was scarce (Table 3.1). We found a low frequency of MRSA (5%, 16/336), mainly among infants (8%, 7/93) and young children (7%, 4/56), followed by toddlers (5%, 4/80) and neonates (1%, 1/104). Resistance to ciprofloxacin was observed in only one MSSA, which presented a MDR profile (CIP-PEN-SXT-GEN). All isolates were susceptible to vancomycin, daptomycin and linezolid. The 317 not susceptible *S. aureus* displayed 32 resistance profiles. Among the 85 MDR strains, the most common profile was PEN-TCY-ERY-CLID (31%), followed by PEN-ERY-CLID (20%) and FOX-PEN-TCY-ERY-CLID-GEN-SXT-CHL (11%). This last profile was the most commonly observed amongst MRSA (56%) (Supplementary Table S3.3). The morphologically distinct *S. aureus* isolated from the same blood culture (same patient) showed distinct resistance patterns, as

follows: (i) PEN *vs*. PEN-ERY-CLID; (ii) TCY *vs*. TCY-ERY-CLID, and (iii) PEN *vs*. PEN-ERY.

Table 3.1. Antimicrobial resistance rate of *Staphylococcus aureus* isolated from children aged <5 years admitted with bacteraemia (n=336).

Antibiotic name	NS [a]		
	R	I	Total
	n (%)	n (%)	n (%)
Penicillin G	304 (90)	0	304 (90)
Cefoxitin	16 (5)	0	16 (5)
Tetracycline	156 (46)	5 (1)	161 (48)
Erythromycin	69 (21)	15 (4)	84 (25)
Clindamycin	69[b] (21)	7 (2)	76 (23)
Co-trimoxazole	32 (10)	4 (1)	36 (11)
Chloramphenicol	15 (4)	1 (<1)	16 (5)
Gentamicin	11 (3)	4 (1)	15 (4)
Ciprofloxacin	1 (<1)	0	1 (<1)

[a]NS, Not Susceptible, includes isolates with Resistant (R) or Intermediate (I) phenotypes; [b] All clindamycin resistance phenotypes observed were inducible. All the *S. aureus* isolates were susceptible to vancomycin, daptomycin and linezolid.

3.4.3. Pattern of antimicrobial resistance over the surveillance period

The antibiotic resistance pattern varied over the two decades of study according to the antibiotic tested (Figure 3.1). The pattern of resistance to penicillin was the most stable throughout the years with rates above 80% and peaking (100%) at the beginning (2004), middle (2011–2013) and at the end of the study period (2015–2019). The resistance rates to tetracycline ranged between 35% and 61% from 2001 to 2009 and steadily reduced (following the low *S. aureus* isolation frequency) in the subsequent years with sporadic peaks. In turn, resistance to erythromycin/clindamycin varied considerably (0%–50%) throughout the entire period of the surveillance, with peaks in 2015 and 2018; while resistance to co-trimoxazole increased between 2002 and 2013 (0%–27%) and was not detected from 2014 onwards. MDR strains accounted for 25% of the *S. aureus* isolated in the first year of surveillance (2001), reaching 40% in 2005. MDR frequency varied considerably over the study period (between 0% in 2016 and 50% in 2013, 2015, and 2018), albeit the

absolute frequency of MDR strains diminished, following the lower isolation of *S. aureus* (Figure 3.2A). MRSA frequency ranged from less than 10% in the first decade of surveillance (2001–2010) to 0% in the second decade (2011–2019), except for 2014 and 2015, in which a single MRSA was detected each year (Figure 3.2B).

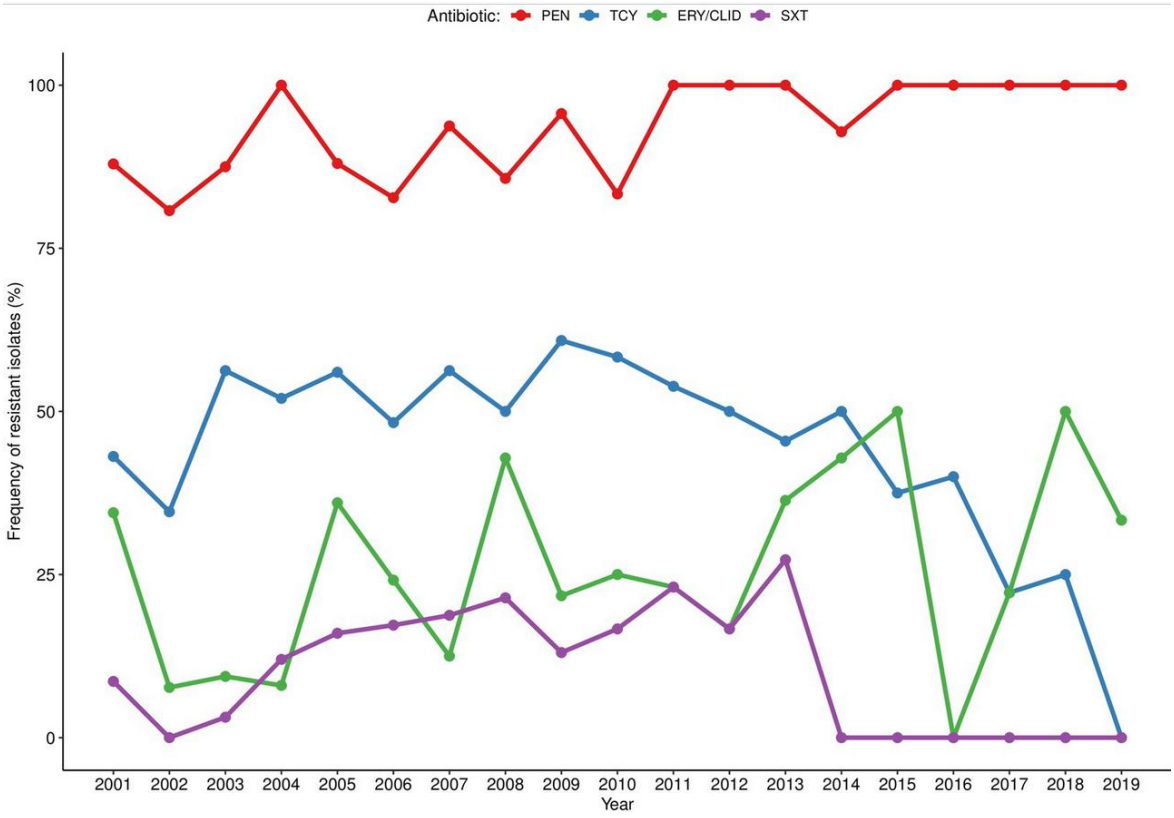

Figure 3.1. Temporal distribution of antibiotic resistance rates among *Staphylococcus aureus* isolated in children with bacteraemia. Resistant strains correspond to those presenting not susceptibility phenotype (resistant or intermediate). PEN: penicillin; TCY: tetracycline; ERY/CLID: erythromycin/clindamycin; SXT: co-trimoxazole.

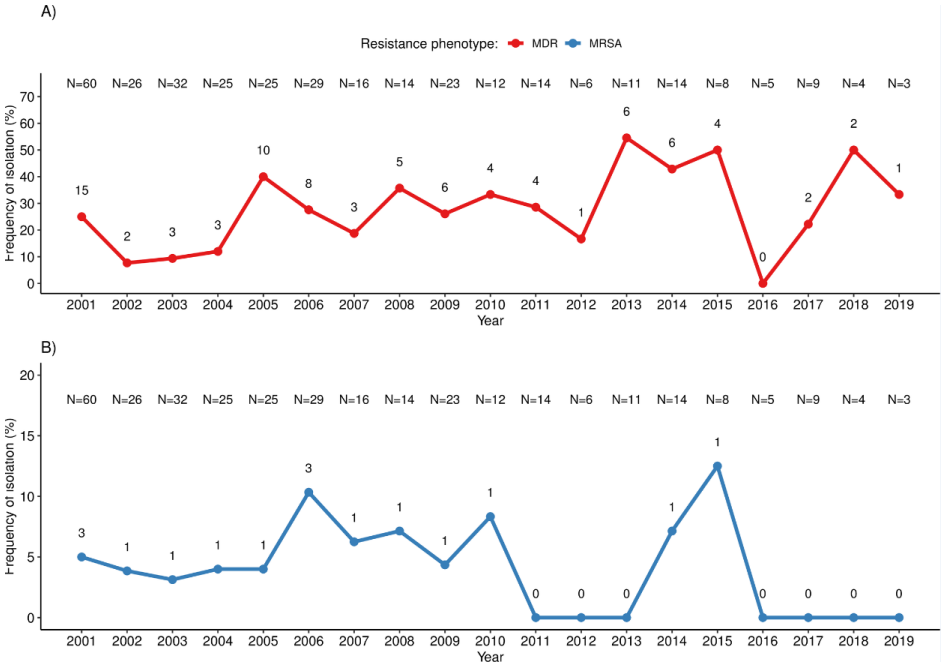

Figure 3.2. Temporal distribution of multiresistant *S. aureus* isolated in children with bacteraemia. (**A**) MDR, multidrug resistant *S. aureus* defined as those not susceptible to ≥ 3 unrelated classes of antibiotics; (**B**) MRSA, methicillin-resistant *S. aureus*. The number in each point *(n)* corresponds to the number of MDR/MRSA strains, while the number at the top *(N)* corresponds to the total number of *S. aureus* related to bacteraemia isolated in that year.

3.4.4. Resistance determinants

A good agreement was found between a not susceptibility phenotype and the resistance determinants screened, with the level of agreement by Cohen's κ test revealing a "substantial perfect agreement" for tetracycline [$\kappa = 0.7$, 95% CI (0.6–0.8)] and "almost perfect agreement" for penicillin and cefoxitin [$\kappa = 0.9$, 95% CI (0.9–1.0) and 1.0, 95% CI (1.0–1.0), respectively] (Supplementary Table S3.4). For instance, total concordance was found between cefoxitin resistance phenotype and genotype, and 303 (>99%) out of 304 strains not susceptible to penicillin carried the *blaZ* gene encoding for a beta-lactamase (Table 3.2). For tetracycline, 157 out of 161 (98%) strains not susceptible to this antibiotic carried at least one *tet* determinant (*tet*(K), *tet*(L) or *tet*(M)), of which twenty-four carried two determinants (*tet*(L)-*tet*(M) ($n = 12$), *tet*(K)-*tet*(L) ($n = 10$), *tet*(K)-*tet*(M) ($n = 2$)) and five strains carried the three genes screened. Regarding co-trimoxazole, 31 out of 36 (86%) strains not susceptible carried *dfrA*(S1) and/or *dfrG* genes (including 2 strains carrying both genes). For

macrolides, 69 out of 84 (80%) strains not susceptible carried *erm*(C) and/or *msr*(A) (including 5 strains carrying both genes), while the *erm*(A) gene was not detected. Two-thirds of the strains resistant to chloramphenicol carried the cat_{pC221} gene. The single strain resistant to ciprofloxacin had mutations in the QRDR of GrlA ([S80F] and [S144P]) and GyrA ([S84A]) targets (Table 3.2).

Table 3.2. Comparison between the susceptibility phenotype and carriage of resistance determinants among the *S. aureus* isolates characterized.

Antibiotic name	Resistance determinants	Susceptibility category n/N (%)		
		Resistant	Intermediate	Susceptible
Penicillin	*blaZ*	303/304 (>99)	NA	1/32 (3)
Cefoxitin	*mecA*	16/16 (100)	NA	0
Tetracycline[a]	*tet*(K)	119/156 (76)	3/5 (60)	9/175 (5)
	tet(L)	29/156 (19)	0/5 (0)	ND
	tet(M)	39/156 (25)	1/5 (20)	ND
Co-trimoxazole[b]	*dfrA*(S1)	12/32 (38)	0/4 (0)	ND
	dfrG	18/32 (56)	3/4 (75)	ND
Erythromycin[c]	*erm*(C)	63/69 (91)	4/15 (27)	ND
	*erm(*A)	0/69 (0)	0/15 (0)	ND
	*msr(*A)	6/69 (9)	1/15 (7)	ND
Gentamycin	*aacA-aphD*	11/11 (100)	3/4 (75)	ND
Chloramphenicol	cat_{pC221}	10/15 (67)	0/1 (0)	ND
Ciprofloxacin	GrlA ([S80F]+[S144P]) + GyrA [S84A]	1/1 (100)	0	ND

NA, Not Applicable; ND, Not determined; [a]Three out of 156 isolates resistant to tetracycline, and 1/5 isolates with intermediate resistance were simultaneously negative to *tet*(K), *tet*(M) and *tet*(L) genes; [b]Four out of 32 isolates resistant to co-trimoxazole, and 1/4 with intermediate resistance were simultaneously negative to the *dfr*A(S1) and *dfrG* genes; [c]Four out of 69 isolates resistant to erythromycin, and 11/15 isolates with intermediate resistant were simultaneously negative to the *erm*(C), *erm*(A) and *msr*(A) genes.

3.4.5. Molecular typing

The *spa* typing revealed a high genetic diversity (SID = 0.97, CI 95% [0.96–0.97]) with 80 different *spa* types found among the entire collection ($n = 336$), including two novel types (t19593 and t19871). The high diversity of the study collection was further confirmed by PFGE analysis (performed for a subset of 160 isolates), revealing that 78% (124/160) of the

isolates typed were clustered in 30 pulsotypes and in 109 subtypes, with each subtype containing one to three isolates, while the remaining 22% (36/160) isolates corresponded to single pulsotypes (data not shown). Frequent *spa* types were t084 (8%) and t002 (7%), followed by t355 (6%), t186 (6%), t645 (5%) and t174 (5%). The remaining *spa* types corresponded to <5% of the isolates (one to fifteen isolates) (Table 3.3). In two cases, the morphologically distinct *S. aureus* isolated from the same blood culture belonged to the same *spa* type (t888 and t934, respectively) while in the third case they belonged to distinct *spa* types (t008 and t174).

A subset of 168 *S. aureus*, representative of different *spa* types, was further analyzed by MLST, revealing 45 distinct STs (SID = 0.95, CI 95% [0.94-0.96]), including sixteen novel ones (ST6098, ST6101, ST6102, ST6304, ST6994, ST6995, ST6996, ST6997, ST7081, ST7082, ST7201, ST7202, ST7349, ST7350, ST7351, and ST7355). ST8 and ST25 predominated (12% and 10%, corresponding to 20 and 17 out of 168 isolates, respectively), followed by ST152 (7%), ST5 and ST612 (7% each), ST15 (6%), ST1 and ST88 (5% each). The remaining STs, including ST6, ST12, ST22, ST72, ST80, ST97, and ST121 corresponded to <5% of the collection (one to seven isolates) (Table 3.3). The novel STs corresponded to single ($n = 12$) or double ($n = 3$) locus variants of other STs circulating in Manhiça district. Forty-three STs clustered within thirteen CCs, while two STs (ST3502 and ST7349) were singletons, defined as those that did not match other STs at ≥4 loci (Figure 3.3). The clonal analysis of the entire *S. aureus* collection (determination of clonal complexes based on MLST for 168 isolates, and inference for the remaining 168 isolates as described in the Methodology), revealed a predominance of CC5 and CC8 (~17%, each), followed by CC15, CC1, CC121, CC152, CC88, CC25, and CC80, with frequency rates varying between 5% and 11% (Table 3.3). CC12, CC22, CC45, and CC97 were represented by <3% of the isolates (one to eight isolates) (Table 3.3).

Table 3.3. Clonal relatedness among *S. aureus* (n = 336) analysed by *spa* typing and MLST.

CC[a]	ST[b]	*Spa* type	MRSA n/N (%)	MDR n/N (%)
CC5 (N=58)	ST5	t002, t010, t071, t105, t306, t5259, t579, t8470	0	6/58 (10)
	ST6	t701, t934, t1476, t2360		
	ST650	t002, t062, t1470, t4535		
	ST6101	t045		
	ST6304	t002		
	ST7081	t304		
CC8 (N=56)	ST8	t008[c], t334, t1476, t5472	15/56 (27)	22/56 (39)
	ST72	t148, t3092		
	ST612	t064[c]		
	ST770	t9045		
	ST7201	t487		
CC15 (N=37)	ST15	t084, t085, t346, t1492, t11928	0	2/37 (5)
	ST1160	t085, t9045		
	ST1906	t4340		
	ST6996	t3370		
	ST7202	t084		
	ST7351	t774		
CC1 (N=36)	ST1	t127, t174, t254, t1931, t5471, t8538, t10719	0	9/36 (25)
	ST188	t2883		
	ST573	t1839		
	ST805	t1476		
	ST1292	t3086		
	ST2139	t174		
	ST7355	t14473		
CC121 (N=34)	ST121	t272, t317, t645, t1114	0	9/34 (26)
	ST2430	t645, t19593, t2793		
	ST6098	t3772		
	ST6102	t14460		
	ST6995	t2793		
	ST7350	t2793		
CC152 (N=33)	ST152	t355, t888, t1096, t1299, t1931, t5047	0	12/33 (36)
CC88 (N=26)	ST88	t186, t690, t1951, t4125, t5351[c], t6449	1/26 (4)	6/26 (23)
	ST2141	t18888		
	ST7078	t786		

[a]CC, clonal complex; [b]ST, sequence type; [c]*Spa* types which included MRSA strains.

Table 3.3. ***(Cont.)*** **Clonal relatedness among *S. aureus* (n = 336) analysed by *spa* typing and MLST.**

CC80 (N=16)	ST80	t376, t934, t12119	0	1/16 (6)
	ST6994	t1198		
	ST7082	t16489		
CC25 (N=20)	ST25	t078, t19871, t2554, t258, t3662, t3772, t9045	0	16/20 (80)
CC22 (N=6)	ST22	t891	0	1/6 (17)
CC45 (N=8)	ST508	t015	0	1/8 (13)
	ST6997	t445		
CC12 (N=3)	ST12	t888	0	0
CC97 (N=1)	ST97	t426	0	0
Singletons (N=2)	ST3502	t2767	0	0
	ST7349	t10294		

[a]CC, clonal complex; [b]ST, sequence type; [c]*Spa* types which included MRSA strains.

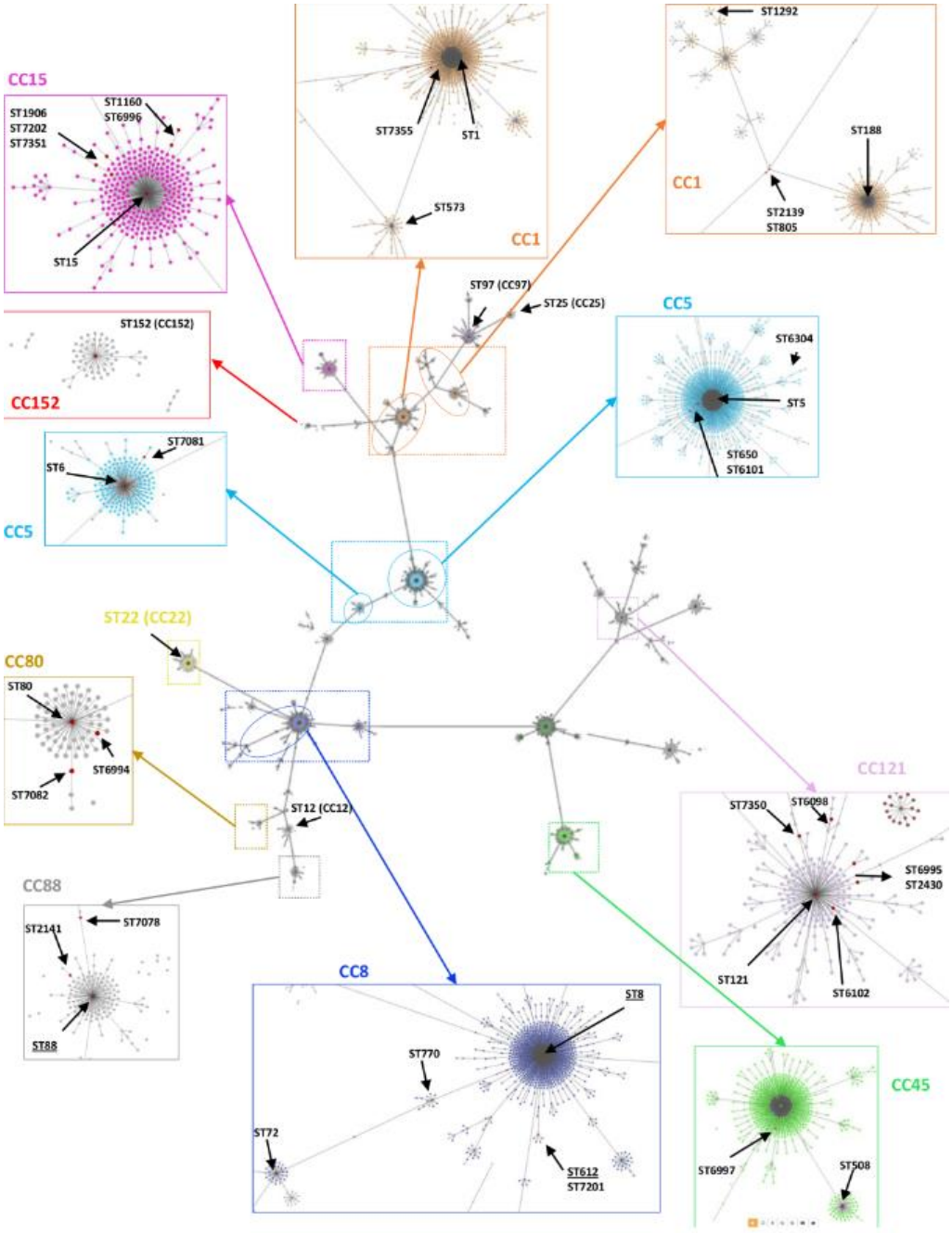

Figure 3.3. Overview of the clonal relatedness among *S. aureus* isolated in children with bacteraemia. The genetic relatedness was determined using PHYLOViZ Online software, including all the STs/CCs found in this current study plus the ones deposited in the PubMLST database until December 2022. Lines link all STs up to triple locus variants. The zoomed colored boxes highlight the clonal complexes (CCs) identified in this study, indicating all STs found by red dots. The underlined STs (e.g., ST8) indicate the ones including MRSA isolates.

3.4.6. SCC*mec* typing

SCC*mec* typing revealed that 12 out of the 16 MRSA (75%) carried a SCC*mec* type IV, which corresponded to SCC*mec* subtype IVa (ST88/CC88) and subtype IVd (ST612/CC8) for one and eleven strains, respectively. The remaining four MRSA (25%, ST8/CC8) carried a non-typable SCC*mec* (SCC*mec*NT).

3.4.7. Temporal distribution of *S. aureus* clones

Considering now the wider picture provided by the analysis of CCs, we observed an overall decrease of CC1, CC5, CC8, CC15, CC25, CC80, and CC88 throughout the surveillance and their absence in the last years (Figure 3.4). An opposing increasing trend was observed for CC121 and CC152, particularly for CC152 that was initially detected in 2001 and resurfaced in 2010 with remarkable increase throughout the remaining surveillance period, becoming the main clonal lineage of the last 6 years (Figure 3.4). Among the MRSA, clone t064-ST612/CC8-SCC*mec*IVd (69%, 11/16) was found only in the first nine years (2001, 2003–2009) of the surveillance, while clone t008-ST8/CC8 (25%, 4/16) harboring SCC*mec*NT was detected sporadically (2001, 2002, 2010, and 2015). A single MRSA strain from clone t5351-ST88/CC88-SCC*mec*IVa belonging to the "African clone" (Schaumburg *et al.*, 2014) was isolated in 2014, thirteen years after the first detection of ST88-MSSA in our surveillance. The single ciprofloxacin resistant strain, which was detected in 2009, presented a MDR phenotype and belonged to clone t891-ST22/CC22. Strains from the same lineage (t891/CC22) but susceptible to ciprofloxacin and with a non-MDR/MSSA phenotype had been previously detected in 2006 (one strain), and then in 2009, 2011, 2016, and 2017 (one strain in each year).

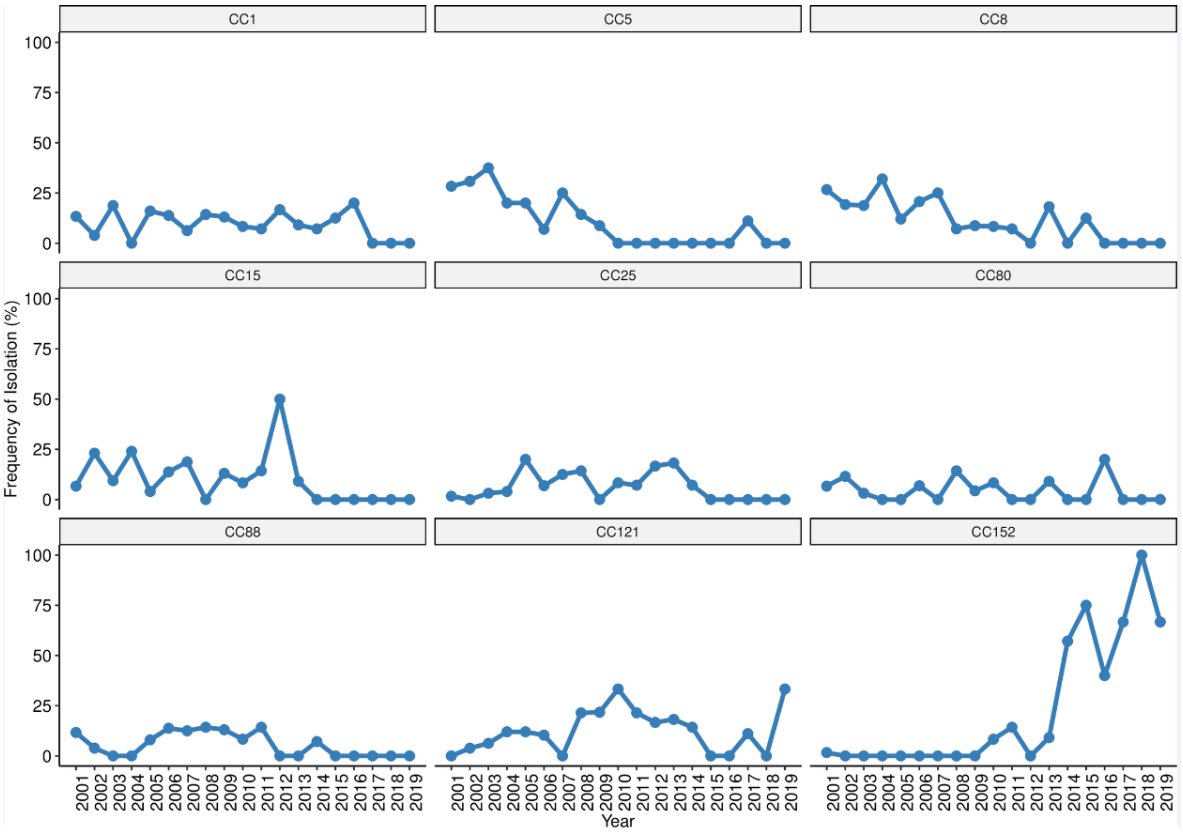

Figure 3.4. Trends of the most prevalent *S. aureus* clonal complexes isolated in children with bacteraemia.

3.4.8. Relatedness between *S. aureus* clonal lineages and antibiotic resistance phenotypes

Our analysis revealed STs containing exclusively MRSA strains [ST612 ($n = 11$)], while others included both MRSA [ST8 ($n = 4$), ST88 ($n = 1$)] and MSSA strains [ST8 ($n = 16$), ST88 ($n = 8$)]. The MDR phenotype was predominantly observed among strains belonging to the ST612 [MDR ($n = 11$) *vs.* non-MDR ($n = 0$), $p < 0.001$] and ST25 [MDR ($n = 13$) *vs.* non-MDR ($n = 4$), $p < 0.001$], while non-MDR phenotype was predominantly or exclusively found among the remaining STs. Overlaying the CC data, MRSA belonged exclusively to CC8 and CC88, while MDR were commonly found among CC25. The MDR phenotype was also found in >35% of the strains from CC8 and CC152, and in >23% of CC1, CC88, and CC121. In contrast, non-MDR were significantly found among CC5, CC8, and CC15 (Table 3.3 and Figure 3.5).

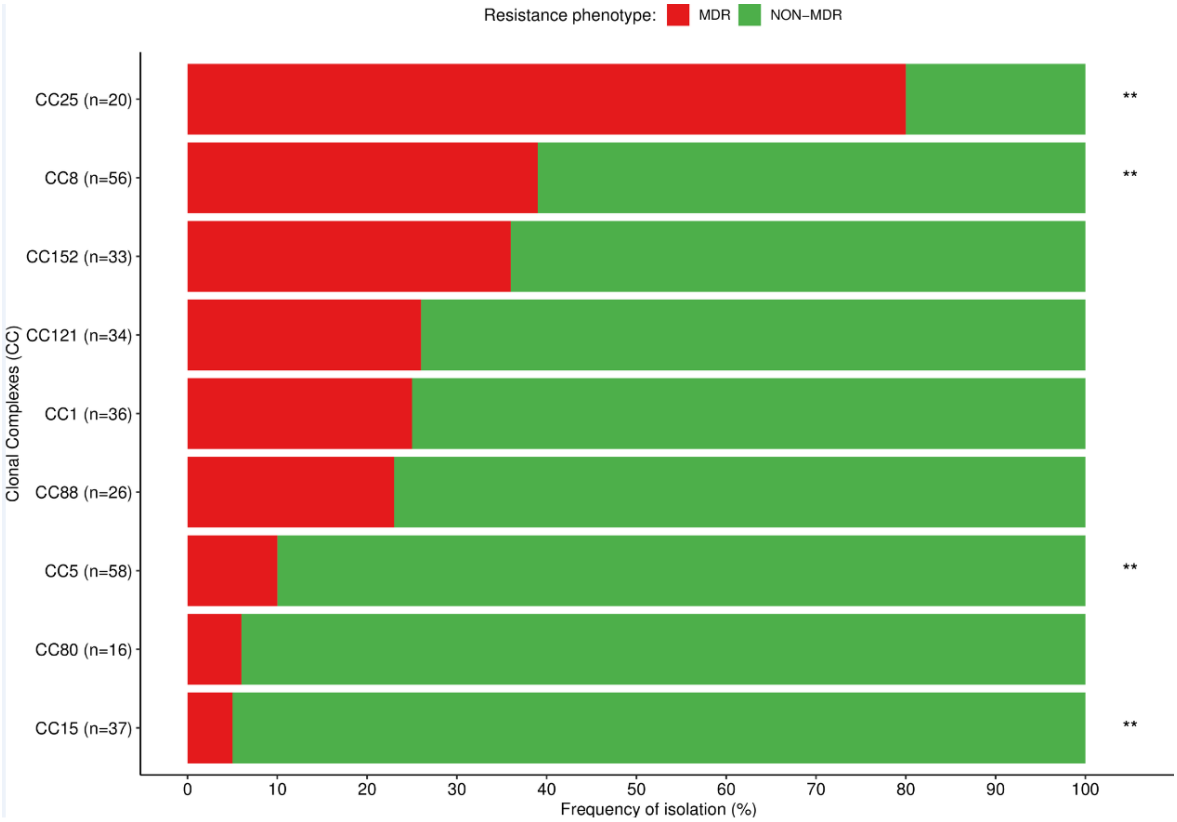

Figure 3.5. Distribution of the most prevalent clonal complexes among MDR (red) and non-MDR (green) *S. aureus* isolated in children with bacteraemia. Differences in the distribution of CCs between MDR and non-MDR isolates were calculated with $\chi 2$ or Fisher's exact test as appropriate; $^{**}p < 0.01$.

Resistance to penicillin and penicillin-tetracycline were frequent among most CCs, while resistance to gentamicin was exclusively found among CC8 and CC22 (Table 4.4). Resistance to co-trimoxazole predominated among members from CC8 and CC25 and was less frequent among the CC1, CC5, CC22, CC88 and CC121. Similarly, resistance to chloramphenicol was highest among the CC8 and was less frequent among CC1 and CC25; while CC80 grouped most strains either fully susceptible or exclusively resistant to tetracycline (Table 4.4).

Table 3.4. Phenotypic resistance and resistance determinants among the most prevalent *S. aureus* clonal complexes.

CC	Resistance patterns (n)	Main Resistance determinants (n)	MRSA n (%)	MDR n (%)
CC5 (N=58)	PEN (31)	*blaZ* (31)	0	6 (10)
	PEN-TCY (14)	*blaZ-tet*(K) (10)		
	Others[a] (8)	*blaZ-tet*(L)-*tet*(M)-*mrs*(A) (1), *blaZ-tet*(K)-*erm*(C) (1)		
	Fully susceptible (5)	*blaZ* (1)		
CC8 (N=56)	PEN (15)	*blaZ* (14)	15 (27)	22 (39)
	PEN-TCY (16)	*blaZ-tet*(K) (13)		
	FOX-PEN-TCY-GEN-SXT-CHL-ERY-CLID (9)	*blaZ-tet*(M)-*mecA*-*erm*(C)-*aacA_aphD*-*dfrA*(S1)-*cat* (5)		
	PEN-TCY-ERY-CLID (5)	*blaZ-tet*(L)-*tet*(M)-*erm*(C) (3)		
	Others[a] (11)	*blaZ-tet(K)-dfrG* (2)		
CC15 (N=37)	PEN (18)	*blaZ* (16)	0	2 (5)
	PEN-TCY (17)	*blaZ-tet*(K) (16)		
	Others[a] (2)	*blaZ-tet*(K)-*erm*(C) (2)		
CC1 (N=36)	PEN (14)	*blaZ* (12)	0	9 (25)
	PEN-TCY (7)	*blaZ-tet*(K) (5)		
	PEN-TCY-ERY-CLID (3)	*blaZ-tet*(K)-*erm*(C)-*msr*(A)		
	PEN-ERY-CLID (3)	*blaZ-erm*(C) (3)		
	TCY (2)	*tet(M)*		
	Others[a] (7)	*blaZ (2)*		
	Fully susceptible (2)			
CC121 (N=34)	PEN-TCY (11)	*blaZ-tet*(M) (7)	0	9 (26)
	PEN (10)	*blaZ* (10)		
	PEN-TCY-ERY-CLID (6)	*blaZ-tet*(M)-*erm*(C) (4), *blaZ-tet*(K)-*erm*(C) (2)		
	PEN-ERY (3)	*blaZ-erm*(C) (2)		
	Others[a] (4)	*blaZ-erm*(C)-*dfrG*, *blaZ-tet(M)-dfrG*		
CC152 (N=33)	PEN (14)	*blaZ* (14)	0	12 36)
	PEN-ERY-CLID (8)	*blaZ (3)*, *blaZ-erm*(C) (3), *blaZ-tet*(K)-		
	PEN-TCY (5)	*blaZ-tet*(K)-*tet*(L) (3)		
	PEN-TCY-ERY-CLID (4)	*blaZ-tet*(K)-*tet*(L)-*erm*(C) (1), *blaZ-tet*(K)-*erm*(C) (1)		
	Fully susceptible (2)			

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin. *Resistance profiles corresponding to ≤ 5% of isolates are detailed in Supplementary Table S3.5.

Table 3.4. ***(Cont.)*** **Phenotypic resistance and resistance determinants among the most prevalent** ***S. aureus*** **clonal complexes.**

C88 (N=26)	PEN-TCY (14)	*blaZ-tet*(K) (13)	1 (4)	6 (23)
	PEN (2)	*blaZ* (2)		
	PEN-ERY (2)	*blaZ* (2)		
	PEN-TCY-ERY-CLID (2)	*blaZ-tet*(L)-*tet*(M)-*erm*(C) *(1)*, *blaZ-tet*(K)		
	Others[a] (6)	*blaZ-erm*(C) (2)		
CC25 (N=20)	PEN-TCY-SXT-ERY-CLID (5)	*blaZ-tet*(K)-*erm*(C)-*dfrG* (5)	0	16 (80)
	PEN-SXT-ERY-CLID (3)	*blaZ-ermC-dfrG* (2)		
	PEN-TCY-SXT (3)	*blaZ-tet*(K)-*dfrG* (3)		
	PEN-SXT (2)	*blaZ-dfrG* (1)		
	Others[a] (7)	*blaZ-tetK-erm*(C) (1), *blaZ-erm*(C)-*cat* (1)		
CC80 (N=16)	TCY (7)	*tet*(K) (7), *blaZ-tet*(K) (1)	0	1 (6)
	PEN-ERY-CLID (1)	*blaZ-erm*(C) (1)		
	PEN-TCY (1)	*blaZ-tet*(K) (1)		
	Fully susceptible (7)			

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin. [*]Resistance profiles corresponding to ≤ 5% of isolates are detailed in Supplementary Table S3.5.

3.4.9. Comparison between clonal lineage and clinical outcome

The comparative analysis of the microbiological data with available clinical records for length of stay, LOS ($n = 279$), revealed that children infected with strains from CC1, CC8, CC15, CC22, CC80, and CC152 had extended LOS (≥5 days) compared to those infected with strains from CC5, CC12, CC25, CC45, CC88, and CC121 (5 days, IQR, 3-8 *vs*. 4 days, IQR, 2-7, respectively, $p = 0.0032$). SAB caused by strains of CC8 was associated with mortality (18%, 9/49 *vs*. 7%, 16/230 for other CCs, $p = 0.023$), while no statistical difference was found for other CCs. Considering the available clinical records, CC8 (49 out of 56 CC8 strains with clinical data) was the only clonal lineage in which infection by MDR strains was associated to mortality compared to non-MDR (4%, 1/28 for non-MDR *vs*. 38% for MDR, 8/21, $p = 0.003$), but no significant difference was found between mortality and infection by MRSA within this specific clone (11%, 4/35 for MSSA *vs*. 36%, 5/14 for MRSA, $p = 0.096$). SAB by CC22 was exclusively found among infants and toddlers, while the CC1, CC5, CC8, CC45, and CC80 predominated among infants; and the CC25 and CC152 were found similarly distributed throughout all the age strata. All CCs but CC152 predominated in the rainy season (69%, 209/301 for other CCs *vs*. 47%, 15/32 for CC152, $p = 0.010$) (Table 3.5).

Table 3.5. *S. aureus* clonal complexes and clinical outcome among children admitted with SAB, stratified by age and rainy season.

***S. aureus* clonal complex (CC[a])**	**Length of hospital admission median days (IQR)[b,c]**	**Case fatality rate by CC[c,d] n/N[d] (%)**	**Age category n/N (%)**				**Rainy season[c] n/N (%)**
			0-28 d	**29 d-11 m**	**12-23 m**	**24-59 m**	
CC8	7 (4 - 10)	9 (36)	15/56 (27)	20/56 (36)	11/56 (20)	10/56 (18)	38/56 (68)
CC80	6 (2 - 8)	0	6/16 (38)	7/16 (44)	2/16 (13)	1/16 (6)	10/16 (63)
CC1	5 (3 - 7)	4 (16)	11/35 (31)	11/35 (31)	8/35 (23)	5/35 (14)	24/35 (69)
CC15	5 (3 - 7)	3 (12)	14/36 (39)	6/36 (17)	13/36 (36)	3/36 (8)	28/36 (78)
CC22	5 (0 - 8)	0	0	4/6 (67)	2/6 (33)	0	4/6 (67)
CC152	5 (2 - 7)	1 (4)	9/32 (28)	7/32 (22)	10/32 (31)	6/32 (19)	15/32 (47)
CC121	4 (3 - 7)	3 (12)	12/34 (35)	5/34 (15)	7/34 (21)	10/34 (29)	21/34 (62)
CC25	4 (3 - 8)	1 (4)	4/20 (20)	7/20 (35)	5/20 (25)	4/20 (20)	13/20 (65)
CC5	4 (2 - 5)	3 (12)	21/56 (38)	13/56 (23)	11/56 (20)	11/56 (20)	39/56 (70)
CC88	3 (2 - 5)	0	7/26 (27)	6/26 (23)	9/26 (35)	4/26 (15)	21/26 (81)
CC45	3 (2 - 4)	1 (4)	3/8 (38)	2/8 (25)	2/8 (25)	1/8 (13)	4/8 (50)

[a] Data of groups with ≤3 isolates were not shown (CC12, CC97, and singletons); [b] IQR: interquartile range; [c]Data from Garrine *et al.*, 2023; [d]Complete records for mortality were available for 279 patients out of 333 children with SAB. d, days, m, months.

3.5. Discussion

3.5.1. High antibiotic resistance rates among bacteraemic *S. aureus*

We performed a comprehensive characterization of the largest collection of SAB-related *S. aureus* strains documented so far in the Manhiça district, Mozambique. Our results showed a high genetic diversity of the *S. aureus* collection and a significant resistance burden, with circulation of 25% of MDR and a few MRSA strains that pose major challenges for the success of antimicrobial therapy in our setting, where the availability of second-line antibiotics is limited. Noteworthy, this study has reported the emergence and predominance of MDR and PVL-positive CC152 MSSA, a clonal lineage prevalent in the European

continent (PVL-positive CA-MRSA), the Caribbean and the African continent (PVL-positive CA-MSSA) (Sowash and Uhlemann, 2014; Baig *et al.*, 2020). Recent studies registered the circulation of PVL-positive CC152 MRSA in regions not previously detected (Democratic Republic of the Congo, Kenya, Nigeria and South Africa) (Lawal *et al.*, 2022).

Data from local studies in Mozambique revealed distinct frequencies of circulating MDR and MRSA strains, with some studies from our setting (Sigaúque *et al.*, 2009; Mandomando *et al.*, 2010; Vubil *et al.*, 2017) and other regions (Ceccarelli *et al.*, 2005; van der Meeren *et al.*, 2014) matching our data, while others reported significantly higher rates (Kenga *et al.*, 2021). Despite the low rate of MRSA in our setting, our findings must be monitored with caution, as countries such as Tanzania, which initially reported a low prevalence of MRSA, saw a subsequent abrupt increase in their incidence (Mzee *et al.*, 2021).

The high rate of penicillin resistance in our study is worrisome as this antibiotic (or ampicillin) in combination with gentamicin are empirically prescribed for hospital admitted patients with suspected invasive bacterial disease in Mozambique. The low resistance rate observed against gentamicin and chloramphenicol (the later less prescribed due to its toxicity, despite occasional use when other antibiotic stock out) suggest that these ready available antibiotics in our setting are still effective against *S. aureus.* Also, our data on MRSA and MDR frequencies supports ceftriaxone as a therapeutic alternative (Garrine *et al.*, 2023). The high resistance rates observed to tetracycline was unexpected, considering its contraindication for administration in children <8 years, and probably reflects the misuse of this antibiotic outside the hospital environment. Previous studies from our setting reported significant proportion of informal antibiotic suppliers (non-licensed providers) (Do *et al.*, 2021), common practice of self-medication and improper storage of medicines for unsupervised reuse (Cambaco *et al.*, 2020). The low resistance rates for tetracycline and co-trimoxazole observed at the end of the surveillance period may reflect the overall reduction of SAB incidence observed by that time (Garrine *et al.*, 2023) rather than changes on antimicrobial susceptibility patterns. Similar rates of resistance towards penicillin and tetracycline were previously reported for *S. aureus* of human or veterinary origin in Mozambique (Vubil *et al.*, 2017; Nhatsave *et al.*, 2021) and other African countries (Kolawole *et al.*, 2013; Seni *et al.*, 2013; Egyir *et al.*, 2014; Eyasu *et al.*, 2015; Dekker *et al.*,

2016; Mekonnen *et al.*, 2018). Contrarily, the resistance to co-trimoxazole in our study was lower (11%) comparing to previous reports for *S. aureus* in our setting (36%–69%) (Sigaúque *et al.*, 2009; Mandomando *et al.*, 2010; Vubil *et al.*, 2017). This difference can reflect the lower number of isolates and shorter period of analysis in those previous reports. Nevertheless, the resistance trend of this antibiotic should be monitored, as co-trimoxazole prophylaxis is one of the key interventions among HIV-infected individuals in resource-limited settings (Saadani Hassani *et al.*, 2015; Ministério da Saúde-Mozambique, 2016), including in Mozambique, where the prevalence of HIV/AIDS is among the highest in the world (Ministério da Saúde-Mozambique, 2021). In addition, sulfadoxine-pyrimethamine, an analogue drug of co-trimoxazole (antifolate drugs) has been extensively used for malaria prevention in HIV-negative pregnant women (WHO, 2013).

Most of the resistance determinants identified in our study are known to be carried in mobile genetic elements. This is an additional point of concern, taking into consideration that these may be transferred between different *S. aureus* strains or between *S. aureus* and other bacteria. Additionally, many of these mobile genetic elements (plasmids, transposons, SCC*mec*) that may carry additional resistance determinants that can build up multiresistance patterns (Haaber *et al.*, 2017; Partridge *et al.*, 2018) and be easily transferred between strains.

3.5.2. Diversity of *S. aureus* circulating in Manhiça District

Our analysis, covering *S. aureus* isolates recovered over two decades of surveillance (2001–2019), revealed circulation of distinct clones, as previously described regionally (Breurec *et al.*, 2011; Schaumburg *et al.*, 2014; Ruffing *et al.*, 2017). The predominant CCs from our study (CC1, CC5, CC8, CC15, CC25, CC121, and CC152) correlated with the ones previously reported in multicenter studies involving several African countries (Breurec *et al.*, 2011; Ruffing *et al.*, 2017). The underlying reasons for the emergence of CC152 and declining of most of others CCs in the last years of the surveillance are still not understood; some studies suggest the competition between different clones and species as one of the factors that favor this expansion in a specific geographic area (Ruffing *et al.*, 2017; Lakhundi and Zhang, 2018). Moreover, the evolution of CC152 mimics in many ways the genotypic and spatial characteristics of the European CC80 CA-MRSA clone, by its emergence from a

PVL-positive MSSA ancestor from North Africa or Europe (Stegger *et al.*, 2014; Baig *et al.*, 2020). The PVL-positive CC152 CA-MRSA was rarely reported outside the European continent, while PVL-positive CC152 MSSA strains was associated with the African continent and the Caribbean, and less often in Europe (Baig *et al.*, 2020). A recent report on the clonal distribution trend of MRSA across 16 African countries revealed overtime dissemination of CC1, CC22, and CC152 not previously found in specific locations (Lawal *et al.*, 2022). Although all CC152 *S. aureus* from our study were MSSA, they should be monitored as a potential emerging CC.

Most MRSA strains in our study belonged to CC8, frequently associated with global outbreaks (Lee *et al.*, 2018), with predominance of t064-ST612/CC8-SCC*mec*IVd and t008-ST8/CC8-SCC*mec*NT reflecting the clonal nature of the MRSA strains circulating in Manhiça. The ST612 is a double locus variant of the major clones USA500/CC8, a HA-MRSA strain (Carrel *et al.*, 2015), and USA300/CC8, an epidemic CA-MRSA (Planet, 2017). The geographical distribution of ST612 is limited, being only described in specific regions of South Africa (Moodley *et al.*, 2010; Jansen van Rensburg *et al.*, 2011; Oosthuysen *et al.*, 2014; Perovic *et al.*, 2015, 2017; Lawal *et al.*, 2022), Tanzania (Moremi *et al.*, 2019) and Australia (Axon *et al.*, 2011; Groves *et al.*, 2016), frequently associated with veterinary practices (Groves *et al.*, 2016; Murphy *et al.*, 2018, 2019; Amoako *et al.*, 2019). On the other hand, clone ST8-SCC*mec*IV has been frequently reported both in hospital and community settings in Angola, Cameroon, Gabon, Ghana, Madagascar, Nigeria, and São Tomé and Príncipe (Abdulgader *et al.*, 2015). The ST88, also known as "African clone" is homogenously distributed across the continent being predominantly MRSA (Schaumburg *et al.*, 2014; Ruffing *et al.*, 2017; Lawal *et al.*, 2022); however, in our study all but one (t5351-ST88-SCC*mec*IVa) of the strains belonging to the ST88 were MSSA. Recent report from our setting revealed circulation of human-adapted strains among *S. aureus* isolated from raw dairy milk samples, raising the hypothesis of potential anthroponotic transmission (Nhatsave *et al.*, 2021). Further studies may include samples from different animal species and farmers in close contact to clarify the transmission dynamics of *S. aureus* between hosts. Despite the declining trend of CC5 (mostly represented by the ST5) in the last years of surveillance in our study, its circulation should be monitored as some studies reported the emergence of

ST5-MRSA through the acquisition of the SCC*mec* element by the ST5-MSSA in Africa (Jansen van Rensburg *et al.*, 2011; Schaumburg *et al.*, 2014). This worrisome clone (ST5-MRSA), has been reported in South Africa (Moodley *et al.*, 2010; Jansen van Rensburg *et al.*, 2011), a border country of Mozambique. We identified novel STs that differed in one to two-point mutations from other STs circulating in Manhiça, suggesting that they evolved from the respective related ancestors. The limitation to type some SCC*mec* may originate on the protocol followed in our study that detects only eight (Zhang *et al.*, 2005) out of fourteen SCC*mec* types and subtypes known to date (IWG-SCC, 2021), or result from the emergence of novel SCC*mec* structural variants.

Overall, the resistance rates were homogenously distributed among distinct CCs in our setting. Noteworthy, some exceptions were observed in which resistance to gentamicin, co-trimoxazole or chloramphenicol were related to specific *S. aureus* clonal complexes, calling for urgent monitoring of its trend. Of concern, these CCs are the ones that included MRSA strains (CC8), quinolone resistant strains (CC22) and significant number of MDR strains (CC25). We reported for the first time in Mozambique the circulation of *S. aureus* ST22 ciprofloxacin resistant carrying mutations in the QRDR of the target GrlA [S80F] and GyrA [S84A] subunits of the DNA Topoisomerase IV and DNA gyrase; in addition to a non-common mutation in GrlA [S144P], suspected to be a genetic polymorphism found both in susceptible and resistant strains (Cabrera *et al.*, 2020). ST22 is one of the most common MRSA lineage healthcare-associated in Europe (Holden *et al.*, 2013; Toleman *et al.*, 2017; Lee *et al.*, 2018), with subsequent spread into the community (Toleman *et al.*, 2017). Therefore, there is a need to extend the ongoing morbidity surveillance to nosocomial infections for early detection and control of main strains of concern circulating in the hospital environment.

3.5.3. Impact of infection by specific CCs in patient outcome

The poor outcome among children infected by CC8 (a clone with global dissemination) in our study, suggests the potential of some clones to cause more severe disease (Recker *et al.*, 2017; Horváth *et al.*, 2020). Additionally, infection by CC8 MDR strains was associated to mortality, possibly in relation to resistance to the first line of empirical treatment (Garrine *et*

al., 2023). This calls for a prompt recognition of SAB by specific clones to allow better clinical management of patients. Future studies should explore the virulence potential of these strains, and their interaction with human and animal hosts.

3.6. Conclusions

We found high diversity of bacteraemic *S. aureus* with high burden of MDR strains posing major challenges for the success of antimicrobial therapy in our setting. Specific clonal lineages were associated with poorer outcomes, in addition to the emergence of important *S. aureus* lineages.

3.7. Ethics statement

The *S. aureus* collection analyzed in this study is in the scope of the ongoing morbidity surveillance system established as part of the CISM´s Health and Demographic Surveillance System approved by the Institutional Ethics Review Board for Health at CISM, and from the National Bioethics Committee for Health. All residents of Manhiça´s district have signed an individual informed consent to become part of the ongoing surveillance.

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CHAPTER 4 - Exploring the virulence potential of *S. aureus* CC121 and CC152 lineages related to paediatric community-acquired bacteraemia in Manhiça, Mozambique

This chapter is based on the research article:

Garrine M, Andrade M, Mandomando, I, Couto, I, Costa SS (2023). Exploring the virulence potential of *Staphylococccus aureus* CC121 and CC152 lineages related to paediatric community-acquired bacteraemia in Manhiça, Mozambique. *Under revision in Sci. Rep.*

Author´s contribution to this work

M. Garrine participated in the study conceptualization, data curation, investigation, methodology, formal analysis, software, visualization, validation, original draft writing, writing-review and editing.

4.1. Abstract

Staphylococcus aureus is one of the most frequent causes of bacteraemia in humans. This bacterium has a variety of virulence traits that allow the establishment and maintenance of infection, such as the Panton-Valentine leukocidin (PVL), encoded by the *lukSF-PV* genes, and their ability to form biofilms. The aim of this study was to characterize *S. aureus* strains causing SAB in children below 5 years of age in the Manhiça district (Southern Mozambique), regarding the presence of PVL-encoding genes and their virulence profile by accessing their capacity to produce biofilms and cause infection in *Galleria mellonella* larvae.

The study collection comprised 336 strains isolated from blood cultures of children admitted to the Manhiça District (Mozambique) between 2001 and 2019, previously characterized regarding antimicrobial resistance profile and clonal lineages. The presence of *lukSF-PV* genes was screened by PCR. Biofilm formation was accessed by the crystal violet adhesion method. The virulence potential of representative strains of relevant clonal lineages was assessed in the *G. mellonella* infection model.

Overall, carriage of PVL-encoding genes was frequent (44%, 147/336) amongst the SAB-related *S. aureus* strains. Yet, an increasing frequency (~70-100%) in PVL-positive strains was registered during the last six years of the two decades surveillance period, which could be linked to the emergence of the CC152 lineage in the setting. Nearly 80% (52/65) of the strains from the emergent clones CC121 and CC152 produced biofilms, although this capacity was enhanced in CC152 strains. CC152, CC121 strains showed a higher virulence potential when compared to the reference strain ATCC 25923, but similar to the virulence potential demonstrated to CC8 strains, a clonal lineage which presented a decreasing frequency trend throughout the study period.

This first study revealed a high frequency of PVL^+ paediatric SAB-related *S. aureus* circulating in Manhiça, Mozambique and the contribution of the emergence of CC152 lineage to carriage of PVL-encoding genes. Additionally, CC152 strains were linked to a stronger capacity to form biofilms. Nevertheless, in the *G. mellonella*, CC152 strains

displayed a similar virulence potential than CC121 or CC8 strains. These results highlight the importance of monitoring CC152-MSSA-PVL$^+$ and other emergent *S. aureus* lineages in the Manhiça district as they display important virulence traits that may impact negatively the management of paediatric *S. aureus* infections in Mozambique.

Keywords: *Staphylococcus aureus*; paediatric bacteraemia, virulence**;** PVL, biofilm**;** *Galleria mellonella*.

4.2. Introduction

Staphylococcus aureus is one of the major causes of bacteraemia and other bacterial infections in humans (Bai *et al.*, 2022a). The versatility of *S. aureus* as a pathogen is determined by a varying repertoire of virulence factors, including toxins that increase the potential of the pathogen to cause a range of diseases (Otto, 2014). Clinically important toxins encoded by *S. aureus* comprise the toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs), exfoliative toxins (ETs), and the Panton-Valentine leukocidin (PVL), encoded by the *lukSF-PV* genes (Ahmad-Mansour *et al.*, 2021; Otto, 2014). PVL is a bi-component exotoxin that forms pores in the membrane of leukocytes, inducing their lysis (Saeed *et al.*, 2018). Although the carriage of *lukSF-PV* has been related to specific types of infections, such as skin and soft tissue infections or invasive infections, the role of PVL in such infections PVL is still controverse (McGuire *et al.*, 2023; Tam and Torres, 2019).

Biofilm production is another important virulence determinant in *S. aureus* (Otto, 2018). Overall, biofilm producing strains are estimated to be associated with 65 to 80% of bacterial infections in humans (Kranjec *et al.*, 2021). Those infections are notorious for their chronicity and resilience against therapy with a consequent increase in morbidity and mortality (Otto, 2018). A better understanding of staphylococcal biofilms is imperative to generate new treatment strategies for biofilm-associated infections and to reduce their significant impact on disease.

Despite the importance of PVL and biofilm on the pathogenesis of *S. aureus*, most of the studies are based on the epidemiology of the disease, on antimicrobial susceptibility

determination or screening of genes encoding specific resistance determinants and virulence factors, with scarce information regarding the *in vitro* or *in vivo* virulence potential of important circulating strains. A few studies have recently evaluated the virulence potential of clinical *S. aureus* (Andrade *et al.*, 2022; Hesketh-Best *et al.*, 2021; Ménard *et al.*, 2021b; Sheehan *et al.*, 2019). These studies were based on infection models that generate *in vivo* data quickly and inexpensively, such as the *Galleria mellonella* larval infection model (Menárd *et al.*, 2021a). The increasing use of this model is supported by its innate immune response which is similar to the one of mammals, including similar mechanisms for pathogen killing (Ménard *et al.*, 2021a; Tsai *et al.*, 2016). However, most of the few available reports determining the virulence potential of *S. aureus* strains are from high and middle-income countries (Andrade *et al.*, 2022; Ménard *et al.*, 2021b; Sheehan *et al.*, 2019).

As the circulating *S. aureus* clonal lineages differ from region to region, studies determining the virulence potential of the main clonal lineages circulating in Africa are needed, particularly those associated with severe disease. We recently reported the epidemiology and clinical characteristics of children with *S. aureus* bacteraemia (SAB) over two decades (2001 - 2019) in the Manhiça district, Mozambique (Garrine *et al.*, 2023a). The *S. aureus* strains (N = 336) were analyzed for their clonality, antibiotic resistance phenotypes and determinants (Garrine et al., 2023a). The data gathered throughout the two decades of study period, revealed an evolution in the predominant clonal lineages overtime. In particular, we observed a predominance of methicillin susceptible *S. aureus* MSSA-CC152 and MSSA-CC121 in the last six years of the study (2014-2019) in contrast to the predominance of CC1, CC5, CC8, CC15, CC25, CC80 and CC88 between 2001 and 2013.

The present study aimed to evaluate the virulence profile of the CC152 and CC121 *S. aureus* related to SAB in our setting. The strains virulence profile was characterized by screening the PVL toxin genes and evaluating the strains capacity to produce biofilms, whereas the virulence potential of representative strains was assessed in the *G. mellonella* larval infection model.

4.3. Methodology

4.3.1. Study design and *S. aureus* collection

In this study we analyzed *S. aureus* strains isolated from blood culture of children admitted to the Manhiça District Hospital – MDH (Manhiça District, Mozambique) between 2001 and 2019. The detailed characteristics of our *S. aureus* collection (N = 336) is presented in Table 4.1. These strains are part of the 24h morbidity surveillance ongoing in MDH as one of the main activities of the *"Centro de Investigação em Saúde de Manhiça"* (CISM). The surveillance performs a standardized collection of clinical data among all paediatric patients (<15 years of age) and a specific microbiological surveillance based on the systematic collection of blood cultures among all admitted patients (Sigaúque *et al.*, 2009).

Table 4.1. Relation between frequency of *lukSF-PV* genes and antibiotic resistance phenotypes of the study collection among the *S. aureus* clonal lineages detected at the Manhiça District Hospital.

Clonal Complex (CC[a])	Sequence type (ST[a])	*lukSF-PV* genes n/N (%)	MRSA[a] % (n/N)	MDR[a] % (n/N)
CC121 (N=34)	ST121, ST2430, T6102	29/34 (85)	0 (0)	9/34 (26)
CC152 (N=33)	ST152	32/33 (97)	0 (0)	12/33 (36)
CC5 (N=58)	ST5, ST650, ST6	23/58 (40)	0 (0)	6/58 (10)
CC8 (N=56)	ST8, ST612, ST72	2/56 (4)	15/56 (27)	22/56 (39)
CC15 (N=37)	ST15, ST1160, ST1906	1/37 (3)	0 (0)	2/37 (5)
CC1 (N=36)	ST1	17/36 (47)	0 (0)	9/36 (25)
CC88 (N=26)	ST88	18/26 (69)	1/26 (4)	6/26 (23)
CC80 (N=16)	ST80, ST6994	10/16 (63)	0 (0)	1/16 (6)
CC25 (N=20)	ST25	7/20 (35)	0 (0)	16/20 80)
CC22 (N=6)	ST22	6/6 (100)	0 (0)	1/6 (17)
CC45 (N=8)	ST508	2/8 (25)	0 (0)	1/8 (13)
CC12 (N=3)	ST12	0 (0)	0 (0)	0 (0)
CC97 (N=1)	ST97	0 (0)	0 (0)	0 (0)
Singletons (N=2)	ST3502, ST7349	0 (0)	0 (0)	0 (0)

[a]Data from Garrine *et al.*, 2023b. MRSA, methicillin-resistant *S. aureus*; MDR, multidrug resistant *S. aureus*.

4.3.2. PCR screening of genes encoding PVL

The three hundred and thirty-six SAB-related *S. aureus* strains were screened by conventional PCR for the presence of *lukSF-PV* genes, encoding PVL. Briefly, the isolates were cultivated in blood agar plates, followed by overnight incubation at 37°C. Afterwards, one colony was transferred to 5 mL of Tryptic Soy Broth (Oxoid Ltd., Basigstoke, UK), and incubated overnight at 37 ºC and 180 rpm. Total DNA was extracted by the boiling method according to an established protocol (Alexopoulou *et al.*, 2006). A 433 bp internal fragment of the *lukSF-PV* genes was screened by PCR, using the primers luk-PV-1 (5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′) and luk-PV-2 (5′-GCATCAASTGTATTGGATAGCAAAAGC-3′) and conditions previously established (Lina *et al.*, 1999).

4.3.3. Evaluation of biofilm production by CC121 and CC152 strains

We evaluated the biofilm mass production for most strains from CC121 (97%, 33/34) and CC152 (97%, 32/33). The strains capacity for biofilm production was assessed by the crystal violet adhesion method (Stepanović *et al.*, 2000), using the conditions described elsewhere (Andrade *et al.*, 2022). Briefly, the isolates were cultured in TSB medium and incubated overnight without shaking at 37 °C. A cellular suspension adjusted to ~ 5 x10^8 CFU/mL in TSB was prepared, diluted 1:100 in TSB supplemented with 1% glucose and 3% NaCl and 0.2 mL aliquots distributed in PS flat-bottom TC 96 well microtiter plates (Orange Scientific, Braine-L' Alleud, Belgium), followed by incubation at 37 °C during 24 h. After incubation, the wells content was discarded and washed with PBS. Adherent bacteria were fixed with methanol (99%) for 20 min and air-dried overnight. The biofilm mass was dyed with crystal violet (0.1%) for 15 min, washed with distilled water and air-dried. The crystal violet was solubilized in acetic acid (33%) for 30 min and the optical density at 570 nm (OD_{570}) was measured in a Synergy HT microplate reader (Biotek, Winooski, VT, USA). Each assay included in quadruplicates, the control strains *S. aureus* ATCC®25923™ (moderate to strong biofilm producer), *S. epidermidis* ATCC®12228™ (weak to moderate biofilm producer, *ica* $^-$), *S. epidermidis* ATCC®35984™ (strong biofilm producer, *ica* $^+$), the *S. aureus* strains under testing, a negative control (supplemented TSB), and a blank (33% acetic acid). Biofilm

production was categorized according to Stepanović criteria (Stepanović *et al.*, 2007, 2000), which establishes a cut-off value (ODc) defined as the geometric mean of OD_{570} of the negative control (supplemented TSB) + 3x the corresponding standard deviation (SD). Strains were characterized as follows: OD_{strain} < ODc, biofilm non-producers; ODc < OD_{strain} < 2x ODc, weak producers; 2x ODc < OD_{strain} < 4x ODc, moderate producers; OD_{strain} > 4x ODc, strong producers. Each assay was performed, at least, in duplicate. To minimize intra- and inter-assay variability, an assay was only validated when associated with an SD value < 20% of the corresponding geometric mean (either considering an individual assay or duplicates) and assigning the strain to the same category. A strain was considered a biofilm producer if assigned to the weak, moderate or strong producer phenotypes.

4.3.4. Assessment of the virulence potential of *S. aureus* in the *G. mellonella* infection model

The virulence potential was evaluated for representative strains of CC121 and CC152, according to the characteristics described in Table 4.2. The *G. mellonella* at the final larval stage were acquired from a pet-food supplier (Reptimundo, Faro, Portugal), maintained in the dark at room temperature and used within two days after arrival. All larval were acclimatized to 37 ºC for 24h before each assay. We determined the virulence potential of each strain using two distinct bacterial inoculums, 10^5 and 10^7 CFU/larva. The procedures for bacterial inoculum preparation and larvae infection were conducted as described elsewhere (Andrade *et al.*, 2022). Briefly, bacteria were inoculated in TSB and incubated overnight at 37 ºC with agitation. The culture was washed in PBS, resuspended and adjusted to ~ 5 x 10^8 CFU/mL, then diluted in 1:100 PBS to achieve ~ 5 x 10^6 CFU/mL and ~ 5 x 10^4 CFU/mL, respectively. Bacteria enumeration was determined by CFU counting. Each assay included four groups of *G. mellonella* larvae (n=10 larvae/group), as follows: (i) non-manipulated; (ii) inoculated with PBS (control); (iii) inoculated with 10^7 CFU/larva; (iv) inoculated with 10^5 CFU/larva. All larvae were inoculated with a 0.02 mL inoculum by injection with an insulin syringe (Omnican 100, Braun) in the last proleg. The *G. mellonella* survival was monitored each 24 h post-infection for seven days, as previously described (Tsai *et al.*, 2016). The virulence potential of each staphylococcal strain was evaluated in, at least,

two independent infection assays (Andrade *et al.*, 2022).

4.3.5. Data analysis

Data from virulence assays in the *G. mellonella* infection model were analyzed by Kaplan-Meier survival curves using GraphPad Prism v8.0.1 (San Diego, CA, USA). Survival rates between groups were compared with the Log-Rank (Mantel-Cox) test. Data on *S. aureus* antibiotic resistance phenotype (multidrug resistant - MDR/non-MDR; methicillin-resistant *S. aureus* - MRSA/MSSA) and clonal lineages (Garrine, M *et al.*, 2023b) as well as the patients outcome (Length of stay, LOS; hospital discharge) (Garrine *et al.*, 2023a) were compared with virulence traits (*lukSF*-PV^{+}, biofilm production phenotype) using χ^2 or Fisher's exact test as appropriate (STATA version 14.2, StataCorp LP, College Station, Texas, USA). We deemed a *p*-value of 0.05 or lower to be statistically significant.

4.4. Results

4.4.1. Frequency of *lukSF-PV* genes among SAB-related *S. aureus* strains

Overall, the *lukSF-PV* genes were detected in 44% (147/336) of *S. aureus* strains. The comparative analysis of the presence of *lukSF-PV* genes and the MLST CCs previously described for this collection (Garrine, M *et al.*, 2023b) revealed predominance of these genes among strains belonging to CC22 (100%), CC152 (97%) and CC121 (85%), followed by CC88 (69%), CC80 (63%) and CC1 (47%) (Table 4.1). The frequency of *lukSF-PV* genes among the remaining CCs varied between 0 and 40%. Analyzing the variation in the frequency of detection of *lukSF-PV* genes throughout the study period, we could observe two distinct trends (Figure 4.1). During the first twelve years of the study (2001 – 2013), the frequency of PVL-positive strains oscillated between ~20% to ~60%. In the following years (2014 – 2019), we observed an increasing trend in PVL-positive strains, accounting for ~70% up to 100% of all SAB-related *S. aureus* strains isolated in that timeframe. Of particular interest, the increasing trend of PVL was linked to the emergence of the CC152 strains.

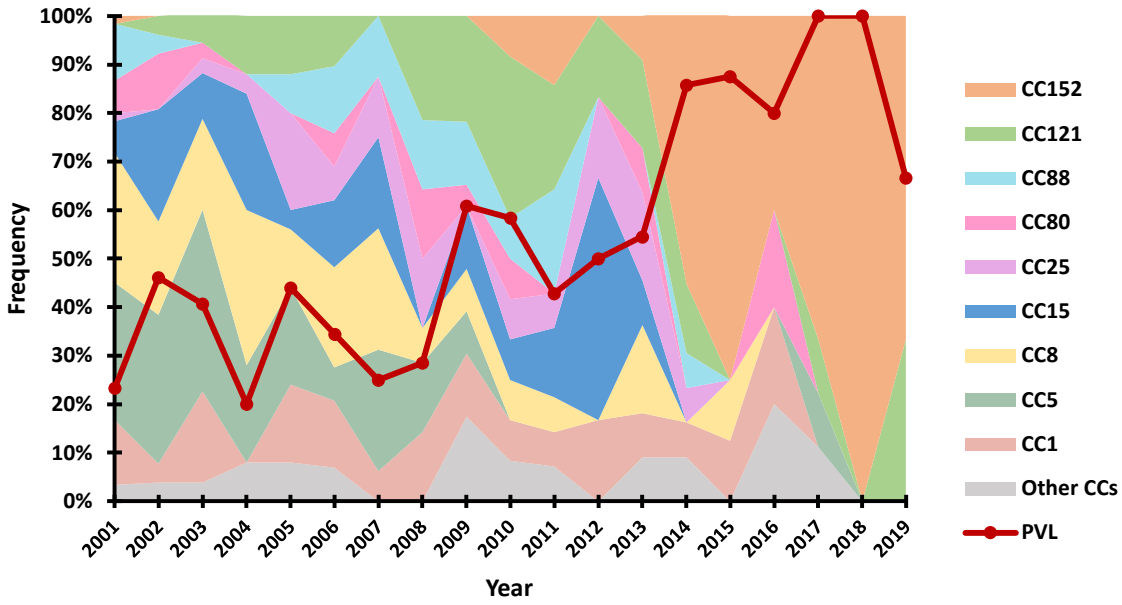

Figure 4.1. Relation between the frequency of *lukSF-PV* genes encoding the Panton-Valentine leucocidin (PVL) and *S. aureus* clonal complexes (CCs) detected at the Manhiça District Hospital throughout the study period. The number of isolates per year of the study period are as follows (2001 - 2019): 60, 26, 32, 25, 25, 29, 16, 14, 23, 12, 14, 6, 11, 14, 8, 5, 9, 4 and 3.

Comparing the presence of *lukSF-PV* genes among MDR and non-MDR strains revealed similar frequencies among both groups (42%, 36/85 for MDR *vs.* 44%, 111/251 for non-MDR, p = 0.764; respectively). Additionally, the *lukSF-PV* genes were not detected among the MRSA isolates. Expanding this analysis to include relevant clinical data (LOS and mortality) (Garrine et al., 2023a), no statistically significant difference was observed between LOS of patients infected by *S. aureus* strains either carrying or not the *lukSF-PV* genes (4 days, IQR: 3-7 *vs.* 5 days, IQR: 3-8, respectively; p = 0.370). No statistical association was also detected between PVL genes carriage and mortality (28%, 7/25 *vs.* 44%, 111/254, respectively; p = 0.129).

4.4.2. Biofilm production

Overall, the capacity to produce biofilms was observed for 80% (52/65) of the CC121 and CC152 strains tested. The frequency of biofilm producing strains (weak, moderate or strong producers) was similar between CC152 and CC121 strains (88%, 28/32 *vs.* 73%, 24/33, respectively; $p = 0.137$). However, considering the level of biofilm production, CC152 strains were significantly more often assigned as strong producers than CC121 strains (78%, 25/32 *vs*. 27%, 9/33, respectively; $p < 0.0001$) (Figure 4.2A). This observation extends to the mean $OD_{570\ nm}$ values observed for the two groups of strains, particularly when comparing strong biofilm producers (Figure 4.2B).

In general, the biofilm production phenotype was not related to MDR phenotypes in either of the clonal lineages studied, even when they were analyzed in combination [CC121+CC152] or separately [CC121 (78%, 7/9 MDR *vs.* 71%, 17/24 non-MDR, $p = 1.000$); CC152 (83%, 10/12 MDR *vs.* 90%, 18/20 non-MDR, $p = 0.620$)]. Similarly, infection by biofilm-producing strains was not related to extended LOS compared to non-producing strains (5 days, IQR: 2-7 *vs.* 4 days, IQR: 3-5, respectively; $p = 0.712$). Also, no association was observed when the LOS was analysed separately for CC121 (5.5 days, IQR: 3-7.5 for biofilm-producing strains *vs.* 4 days, IQR: 3-4 for non-producing strains, $p = 0.464$)) and CC152 (4 days, IQR: 2-7 for biofilm-producing strains *vs.* 5 days, IQR: 3-7 for non-producing strains, $p = 0.859$). The frequency of *lukSF-PV* genes was similar between biofilm-producing and non-producing strains (92%, 48/52 among biofilm producers *vs.* 85%, 11/13 among non-biofilm producers, $p = 0.591$), even when analysed in separate for CC121 (88%, 21/24 among biofilm producers *vs.* 78%, 7/9 among non-biofilm producers, $p = 0.597$) and CC152 (96%, 27/28 among biofilm producers *vs.* 100%, 4/4 among non-biofilm producers, $p = 1.000$).

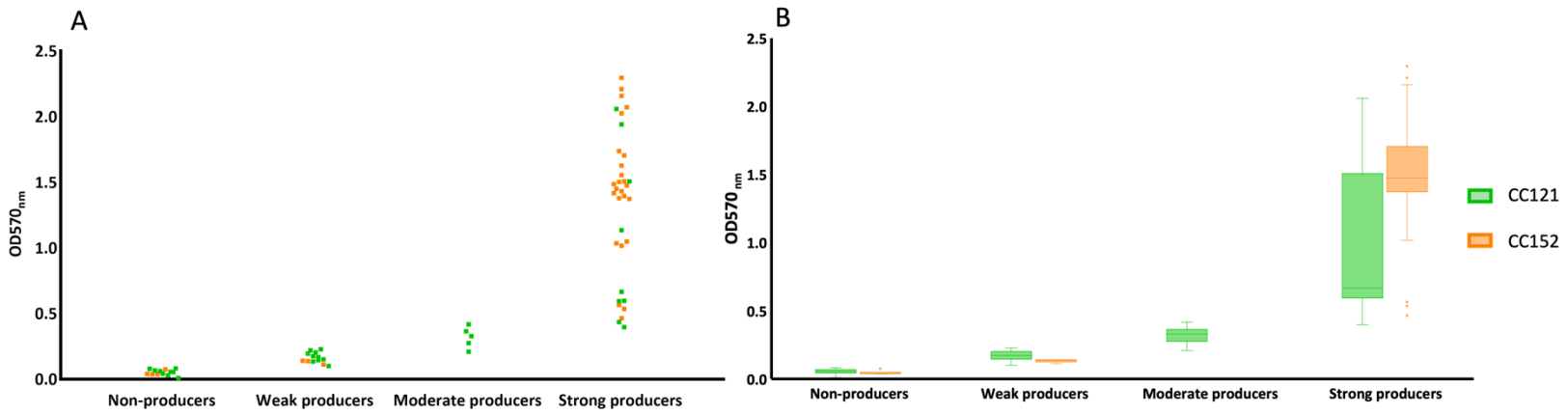

Figure 4.2. Distribution of CC152 and CC121 strains according to their biofilm production phenotype (A) and their variation and median of final OD_{570} nm values (B).

4.4.3. Virulence potential of representative CC121 and CC152 *S. aureus* strains in *G. mellonella* infection model

The virulence potential of nine representative strains of the emergent lineages circulating in our setting, CC121 and CC152, was assessed in the larval *G. mellonella* infection model. Overall, all clinical strains tested showed high virulence in *G. mellonella* when compared to the reference strain *S. aureus* ATCC®25923™. Nevertheless, we could observe a variation of the virulence potential within and between each CC analyzed. Comparing CC121 strains with the reference strain, SA 152 was the most virulent, followed by SA 199 > SA 179 > SA 243 (Figure 4.3). Similarly, CC152 strains presented higher virulence potential than the reference strain, with SA 301 positioned as the most virulent, followed by SA 330 > SA 343 > SA 353 > SA 324. For all strains, the median survival time was inversely proportional to the bacterial inoculum; from 1 to 3.5 days at 10^5 CFU/larva to only 1 day at 10^7 CFU/larva (Table 4.2).

When analyzing the overall virulence potential of *S. aureus* lineage, no relation was found with the virulence traits studied (carriage of *lukSF-PV* genes or biofilm production phenotype) or with the patient outcome (i.e. higher virulence potential did not correlate with poorer patient outcome, and vice-versa) (Table 4.2). Nevertheless, for CC121, non-producing and weak-producing biofilm strains (SA 179 and SA 243, respectively) were less virulent compared to the moderate- and strong-producer strains (SA 199 and SA 152, respectively) (Table 4.2).

We also analyzed the virulence potential of an additional set of four strains, representative of CC8 clonal lineage. Although detected in lower frequency, this lineage comprises the majority of the MRSA strains detected in our setting over the two decades of the study period (15 out of 16 MRSA strains) (Table 4.1). The analysis of the CC8 strains (SA 118, SA 215, SA 276 and SA 277) of the two STs (ST8 and ST612) showed a higher virulence potential than the reference strain and that SA 215 (ST8-MSSA) and SA 277 (ST8-MRSA) were the most virulent, followed by SA 276 (ST612-MRSA) and SA 188 (ST612-MRSA) (Supplementary Figure S4.1). In general, the CC8 strains showed similar virulence potential to the CC121 and CC152 strains.

Table 4.2. Main virulence and antibiotic resistance traits of representative SAB-related *S. aureus* strains selected for the assays in the *Galleria mellonella* infection model.

Strain ID	Clonal Complex[a]	PVL encoding genes	Biofilm production phenotype	Mean OD_{570} value	Median survival time (days)				Resistance profile[a]	Patients outcome[b]
					10^5 inoculum	*p*-value vs. 10^5 ATCC25923	10^7 inoculum	*p*-value vs. 10^7 ATCC25923		
SA ATCC 25923	CC30 (ST243)	+	Strong Producer		5		1		-	-
SA 152	CC121 (ST121)	+	Strong Producer	1.134	1	**0.0003**	1	**0.0374**	Non-MDR	Survived
SA 179	CC121 (ST121)	+	Non-Producer	0.030	2	0.0754	1	**0.0374**	Non-MDR	Died
SA 199	CC121 (nd)	+	Moderate Producer	0.209	2	**0.0025**	1	**0.0374**	MDR	Died
SA 243	CC121 (nd)	+	Weak Producer	0.144	3	0.4255	1	**0.0374**	MDR	Survived
SA 301	CC152 (ST152)	+	Non-Producer	0.037	1	**< 0.0001**	1	**0.0374**	non-MDR	Survived
SA 324	CC152 (ST152)	+	Strong Producer	2.024	3.5	0.1182	1	0.2810	fully susceptible	Survived
SA 330	CC152 (ST152)	+	Strong Producer	2.158	2	**0.0033**	1	**0.0374**	MDR	Survived
SA 343	CC152 (nd)	+	Non-Producer	0.038	2	**0.0042**	1	**0.0374**	non-MDR	Died
SA 353	CC152 (ST152)	+	Strong Producer	0.535	2	0.0929	1	0.1332	MDR	Survived

[a]Data from Garrine *et al.*, 2023b; [b]Data from Garrine *et al.*, 2023a; MDR, multidrug resistant *S. aureus* (defined as those strains not susceptible to three or more unrelated classes of antibiotic). [c]Fully susceptible to the tested antibiotics (Garrine *et al.* 2023b). Significant differences are highlighted at bold type. nd: not determined.

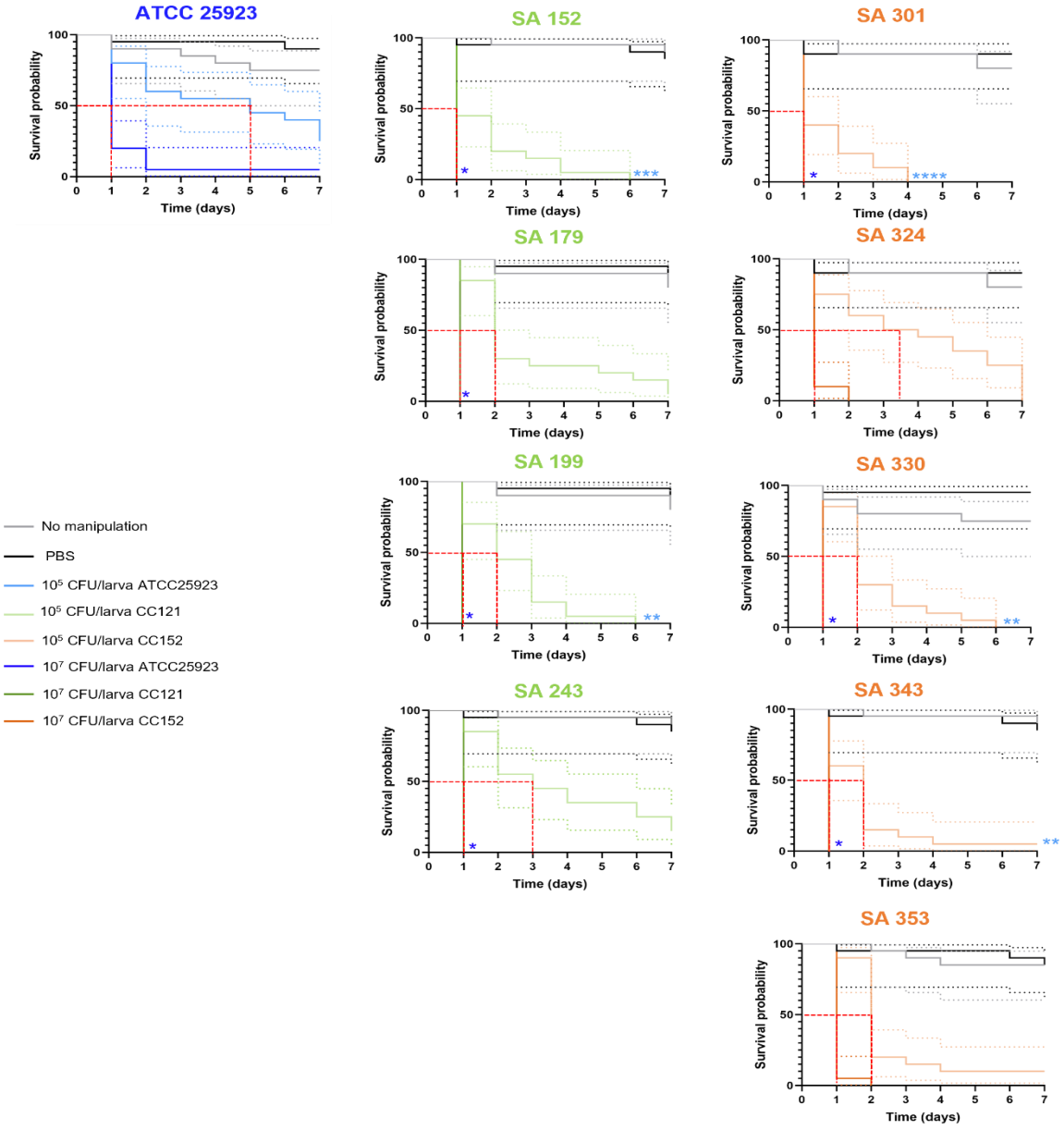

Figure 4.3. Kaplan-Meier survival analysis of *G. mellonella* infection assays with *S. aureus* ATCC25923 (in blue) and SAB-related *S. aureus* strains representative of clonal lineage CC121 (in green) and CC152 (in orange). **The dotted lines indicate the 95% confidence intervals and the red lines indicate the median survival time. Statistically significant differences between each strain and *S. aureus* ATCC25923 (at corresponding inoculums) are identified as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.**

4.5. Discussion

We recently reported the high diversity of *S. aureus* clonal lineages from children with bacteraemia admitted at the Manhiça District Hospital, Mozambique with predominance of the CC121 and CC152 (emergent clone) in the last years of the surveillance system established by CISM in this setting (Garrine et al, 2023b). The same clones were recently observed circulating in other African countries (Lawal *et al.*, 2022), suggesting an important role of these clonal lineages in the epidemiology of staphylococcal invasive infections in this region.

This is one of the few studies assessing the virulence potential of SAB-related *S. aureus* in African countries. Despite the absent correlation between the strains virulence traits analyzed and the survival time of *G. mellonella*, a higher virulence potential was established for the SAB-related *S. aureus* strains circulating in the Manhiça District, compared to the reference strain, particularly for strains from the emergent CC152 lineage. The same pattern of virulence potential was detected for other clinical strains from our collection when compared to the reference strain (Figure S4.1). Several virulence factors carried in *S. aureus* are often encoded on the pathogen's accessory genome. The clinically most important toxins encoded by *S. aureus* are the staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins (ETs) and PVL, a toxin encoded by the *lukSF-PV* genes and responsible for necrotic lesions involving skin and mucosa, and necrotic hemorrhagic pneumonia (Ahmad-Mansour *et al.*, 2021; Otto, 2014). Almost half of the entire *S. aureus* study collection (n=336) carried the *lukSF-PV* genes and these predominated among specific clonal lineages, suggesting potential distinct virulence among distinct clones, as previously suggested (He *et al.*, 2018; Otto, 2010). In our study these genes predominated among CC121 and CC152 and CC22 lineages, supporting the observation of previous reports (Lawal *et al.*, 2022; Ruffing *et al.*, 2017).

Furthermore, the absence of a relation between biofilm production, PVL presence, resistance profile and the virulence potential of either CC121 or CC152 strains in *G. mellonella* infection model, suggests a contribution of additional virulence factors. Noteworthy, in our study, *lukSF-PV* genes predominated among both MDR and non-MDR strains and also in MSSA, and were absent among MRSA strains. In a recent report analyzing the same

collection of *S. aureus* strains, infection by MDR strains was associated to higher mortality of SAB patients (Garrine *et al.*, 2023a). In the present study, infection of *G. mellonella* by MDR strains did not yield a significant difference in the median survival time when compared with infection by non-MDR strains. In agreement with our findings, previous reports described mortality of *G. mellonela* larvae infected with *S. aureus* to be dose-dependent, in which infection with 1×10^7 CFU/larva resulted in death after 24h, whereas infection with 1×10^5 CFU resulted in ∼20% of deaths after 120h (Desbois and Coote, 2011). Our results revealed a high virulence potential of the CC152 lineage predominantly circulating in Manhiça. This finding is matter of concern since CC152 has been reported as an important emergent and prevalent clone in several African countries (Lawal *et al.*, 2022; Baig *et al.*, 2020). Further detailed epidemiological and molecular analysis of this important clone is urgently needed for prompt adoption of prevention and control measures. CC121 for which we also found a high virulence potential, is a clonal lineage also increasingly identified in the African continent (Lawal *et al.*, 2022; Rao *et al.*, 2015).

4.6. Conclusions

To our knowledge, this is one of the first studies documenting the virulence potential of SAB-related *S. aureus* from Mozambique. Here we report a high frequency of MSSA but not MRSA strains carrying the determinant for *lukSF-PV* genes toxin. Strains from the lineage CC152 and, in a lesser extent, CC121, that have established in our setting in recent years are frequent strong biofilm producers and show higher virulence potential in the *G. mellonella* infection model when compared to a prototype reference strain. Although no correlation could be established between particular clonal lineages and virulence, the approach described in this study has potential to be applied to large datasets in future studies.

Our results highlight the importance of monitoring emergent CC152-MSSA-PVL$^+$ and other lineages strains as they display important virulence traits that may impact negatively on the management of SAB paediatric patients in Manhiça, Mozambique.

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CHAPTER 5 – General Discussion and Conclusions

In this Thesis we described the epidemiological and molecular traits of *S. aureus* associated bacteraemia among children under five years of age admitted to the referral hospital of the Manhiça district, Southern Mozambique, over two decades (2001-2019). Overall, we observed that despite the declining rates of SAB incidence during this period, SAB remains an important cause of death among children admitted to MDH, possibly in relation to resistance of the SAB-related *S. aureus* to the first line of empirical treatment in use in our setting (Chapter 2). The detailed molecular characterization of the SAB-related *S. aureus* revealed a high genetic diversity and a significant resistance burden, with circulation of 25% of MDR strains that pose major challenges for the success of antimicrobial therapy in our setting, where the availability of second-line antibiotics is limited. MRSA strains were rare (5% overall) and occurred only throughout the first decade of surveillance (2001-2010) (Chapter 3).

5.1. Epidemiology of SAB in the Manhiça District

The HDSS includes the collection of blood culture upon hospital admission for children <15 years according to previously described criteria (Sigaúque *et al.*, 2009). For this Thesis, we focused the analysis on children aged below five years, as 95% of MDH patients with SAB belonged to this group. Overall, *S. aureus* contributed to 12% of bacteraemia episodes detected in MDH between 2001 and 2019 for this age group, representing a significant burden of bacteraemia in our setting (Figure 5.1).

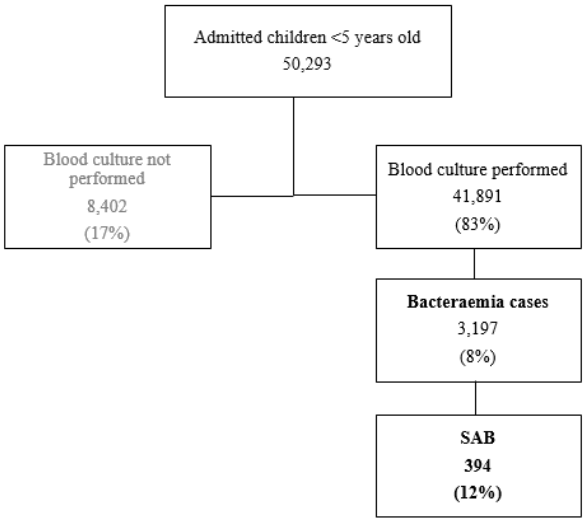

Figure 5.1. Study population and positivity of *S. aureus* bacteraemia among children admitted at the MDH, 2001 - 2019.

The decline of SAB incidence observed throughout the study period (Chapter 2) could be underestimated, considering that (i) only 39.7% (n=157) of the SAB cases detected corresponded to children living within the Manhiça surveillance area, and that only those contributed for incidence estimates; (ii) these estimates included mainly severe cases for which health care was sought (passive detection). Despite this underestimation, the overtime descending trend of SAB observed was similar to the ones for other invasive bacterial infections and clinical malaria previously reported in our setting (Mandomando *et al.*, 2015; Sigaúque *et al.*, 2018, 2013) and other African countries (Mayanja *et al.*, 2010). These include the reported decline of invasive Nonthyphoidal *Salmonella* (iNTS) incidence, from 207.6 cases in 2001 to 20.3 cases per 100,000 CYAR in 2014 and of clinical malaria, from 210.0 cases in 2001 to 12.0 cases per 100,000 CYAR in 2014 (Mandomando *et al.*, 2015). Similarly, the incidence rate of invasive Hib and invasive pneumococcal disease declined in our setting, even before the introduction of the corresponding vaccines (Hib conjugate vaccine and 10-valent pneumococcal conjugate vaccine-PCV10, respectively) into the Mozambique national immunization expanded program (Sigaúque *et al.*, 2018, 2013).

Multiple factors may have contributed to the downward trends of invasive bacterial diseases in Manhiça district, including improvement of socioeconomic, nutritional, sanitary and

hygiene conditions, and also of HIV treatment. Previous reports showed an association between improvement on access to antiretroviral treatment for HIV and declined burden of invasive bacterial diseases among HIV-infected children (Kapogiannis *et al.*, 2008; le Roux *et al.*, 2011; Nunes *et al.*, 2011). High burden of SAB was observed among neonates and infants (Chapter 2), perhaps as a result of the relatively immature immune system (Nunes *et al.*, 2011). Similarly, to our setting, highest incidence of SAB among neonates was recently reported in Gambia (Odutola *et al.*, 2019). Despite the high prevalence of HIV in our surveillance area among adults (39.9%) and pregnant women (23.6%) (González *et al.*, 2012; Menéndez *et al.*, 2008), the burden of HIV infection is not well characterized among children; therefore, the association between HIV-infection and bacteraemia could not be evaluated in our study. Future studies should address the burden of HIV status and its contribution as a risk factor for bacteraemia among children living in the Manhiça district.

5.2. Bacteraemia by *S. aureus*, co-infections and outcome

Most clinical signs and symptoms analyzed are non-specific to SAB and frequently found among bacteraemia caused by other invasive pathogens (Chapter 2), challenging the differential diagnosis based on clinical presentation.

Previous studies in our setting have not significantly associated malaria with bacteraemia, despite its endemicity (Mandomando *et al.*, 2009; Sigaúque *et al.*, 2009). In contrast, this study showed an association between clinical malaria and SAB by multivariate analysis, and a significant association between mortality and SAB in children co-infected with other bacterial pathogens (Chapter 2), calling for an early and accurate diagnosis of these co-infections for a proper inpatient clinical management and reduction of mortality. Infection by malaria is a risk factor for bacteraeamia, as this causes the (i) exacerbation of proinflamatory cytokines and neutrophilic disfunction (Moreira *et al.*, 2021), and (ii) hemolysis (malarial hemolysis) that allows iron availability with consequent bacterial growth; rendering the host susceptible to bacteraemia (Takem *et al.*, 2014).

The case fatality rate observed in our study (9.3%) was similar to the one previously reported for paediatric SAB in our setting (6.0%) (Sigaúque *et al.*, 2009) and in neighboring countries, namely South Africa (8.8%) (Naidoo *et al.*, 2013). However, the mortality here reported

could be underestimated, taking into account that the minimum hospital case fatality rate was calculated considering children with known outcomes at discharge and excluding those patients who left the hospital without medical permission (absconders) or who were transferred to Maputo Central Hospital. SAB is associated with a mortality rate up to 27% in the first three months after diagnosis (Bai *et al.*, 2022b). Therefore, if we assume that children with unknown outcome (absconded [n=29], transferred [n=32], or not reported [n=1]) had worse outcome in our study, the case fatality would reach up to 24%). In addition, data of verbal autopsy in our setting revealed that 50% of local paediatric deaths occur at home, with no previous hospital attendance (Sacarlal *et al.*, 2009). Noteworthy, *S. aureus* has been reported as an important contributor in the mortality among neonates (4%) and children (9%) in the ongoing CHAMPS project, aimed to track definitive causes of death in south Asia and sub-Saharan Africa, including Mozambique (Bassat *et al.*, 2023; Taylor *et al.*, 2020).

Overall, 58% (18/31) of deaths reported in our study occurred early on admission (< 48 hours) (Chapter 2). Also, data from CHAMPS revealed significant mortality rates (36%) observed in the first 24 hours after admission in our setting (Taylor *et al.*, 2020). Those findings suggest the need for urgent and appropriate clinical management of children within the first hours of admission. This is reinforced by the recent CHAMPS data reporting that better clinical management and improved health-seeking behavior, including access to health care could have prevented 47% and 25% of observed deaths; respectively (Bassat *et al.*, 2023). Based on this, CISM is in preparation to participate in the EChiLiBRiST network (Mozambique, Ethiopia and Gabon) to develop and validate a rapid test (based on biomarkers) for early diagnosis and prompt treatment of febrile children at risk of severe disease and death. The methodological framework developed in this Thesis could be applied to the detailed characterization of *S. aureus* isolates from the CHAMPS network, that is urgently needed to enhance the understanding of *S. aureus* epidemiology, clonality and virulence profile in sub-Saharan Africa.

The study collection corresponded to community-acquired infections as the samples were collected upon admission. The current onsite surveillance protocol does not collect

information regarding source of infections, limiting our analyses of risk factors (Chapter 2) or primary site of infection for SAB. On the other hand, CHAMPS data have reported a significant burden of nosocomial infections in the chain of mortality in children under 5 years, mostly due to infection by *Klebsiella pneumoniae* or *Acinectobacter baumannii*, suggesting the need of more comprehensive studies of nosocomial infections in our setting to understand the role of additional pathogens in nosocomial infections (including *S. aureus*) and related risk factors, and how they relate with pathogens associated with community-acquired infections (bacteraemia and SSTIs). The study of those nosocomial infections may be a future follow-up of this work.

5.3. Antimicrobial resistance and clinical outcome

As described in Chapter 3, we observed an association between infection by MRSA and/or MDR strains and mortality, as well as an extended length of stay among MRSA-infected patients. These findings highlight the importance of prompt laboratorial diagnosis and antibiotic susceptibility results to timely guide antibiotic therapy. Persistent SAB and high mortality among patients infected by MRSA compared to MSSA was previously reported (Coombs *et al.*, 2016; Hawkins, 2007; Inagaki *et al.*, 2019; Loftus *et al.*, 2022), likely due to inappropriate empirical antibiotic therapy (Wi *et al.*, 2018). Thus, identifying children infected by MDR or MRSA strains will allow a better guidance for empiric antibiotic therapy and likely improved survival. A recent prospective cohort study conducted at 17 European centres (UK, Spain, and Germany) reported the second day bacteraemia after initiation of active antibiotic treatment as a significant cut-off distinguishing surviving from non-surviving patients (Kuehl *et al.*, 2020). That study suggested 24 h follow-up blood cultures for timely identification of patients with increased risk of death and earlier assessment of additional diagnostic and therapeutic measures (Kuehl *et al.*, 2020), including prolonged courses of treatment (Mathé *et al.*, 2023). Unfortunately, due to financial constraints, the surveillance system at MDH collects only a single blood culture per patient at the time of admission, hampering our ability to assess the persistence of infection. Nevertheless, patient clinical evolution is reassessed every day and antibiotic therapy adjusted if needed, based on *in vitro* antimicrobial resistance data.

The analysis of antibiotic prescription showed that gentamicin, ampicillin, ceftriaxone and penicillin were the most commonly used in our setting, with ceftriaxone showing increased prescription rate throughout the years (Chapter 2). Although penicillin was not prescribed for SAB patients since 2015, ampicillin/amoxicillin, analogues of penicillin, are still in use in our setting in combination with gentamicin. We observed low resistance rate of SAB-related *S. aureus* to cefoxitin (MRSA strains) and to gentamicin (<6%), but a high resistance rate to penicillin (90%) (Chapter 3). The resistance observed to penicillins may challenge the empirical therapy based on combination of ampicillin/penicillin plus gentamicin, while ceftriaxone is still a valuable alternative for empirical therapy. Unfortunately, despite the high rates of resistance reported, penicillin, ampicillin and amoxicillin are still used on empirical treatment in Mozambique and other low income countries with similar burden of resistance, mostly due to the costs for acquisition of alternative/second line antibiotics for empirical treatment. We hope our data provides enough evidence to suggest the reviewing of the current policy recommendations. According to the Mozambican national guidelines, the empirical antimicrobial therapy is started upon admission for children with suspected bacterial infection using parenteral combination of penicillin plus gentamicin for older children. For those children aged < 2 years and other age groups with clinical severe malnutrition the treatment consists of parenteral combination of ampicillin plus gentamicin. Antibiotic therapy is reassessed based on the patient clinical evolution and blood culture results; in which ceftriaxone is used in case of infection by MDR strains or severity of the disease (Glisser, 1997). Patients infected by MRSA or not showing health improvement days after admission are referred to the Maputo Central Hospital (MCH), which is located 80 km of MDH and has availability of vancomycin for treatment of patients infected by MRSA strains. Vancomycin remains the last resort antibiotic for MRSA infections in many countries, with vancomycin-resistant strains (VRSA) occurring sporadically but not spreading due to the strongly increased fitness cost that is imposed by vancomycin resistance genes (McGuinness *et al.*, 2017). Noteworthy, all *S. aureus* collected were susceptible to vancomycin, daptomycin and linezolid (Chapter 3).

We observed that children treated with antibiotics were likely to stay longer at the hospital than those not receiving antibiotic treatment suggesting that the first group presented more

severe illness (Chapter 2). However, due to the lack of complete information on the days of treatment, number of doses of antibiotic given to each child or eventual alterations in therapy, we were unable to assesses the quality and appropriateness of antimicrobial therapy, and relate it to patient's outcome in our setting.

5.4. Phenotypic antimicrobial resistance and resistance determinants

The resistance phenotypes towards distinct antibiotics matched with the resistance determinants screened, where the MRSA phenotype was encoded by *mecA* gene, located in a chromosomal cassette (SCC*mec*) (Haaber *et al.*, 2017). Interestingly, all strains with typable SCC*mec* carried a type IV cassette, which is the simplest one, carrying solely the *mec* complex, *ccr* gene complex and J regions. This type is found both in hospital-acquired MRSA and community-acquired MRSA. In our study, the single isolate carrying SCC*mec* subtype IVa belonged to ST88 (CC88), while all strains carrying SCC*mec* subytpe IVd belonged to the ST612 (CC8). The four strains carrying a non-typable SCC*mec* also belonged to CC8 but ST8 (Chapters 3). We observed different antibiotic resistance patterns among strains carrying SCC*mec* subtypes IVa *vs.* IVd (with additional resistance to co-trimoxazole and chloramphenicol) (Chapter 3), with most deaths reported in patients infected by strains with SCC*mec* subtype IVd, perhaps due to higher virulence (Chapter 4). The reason for distinct phenotypes even for strains with subtypes (IVa *vs.* IVd) from the same SCC*mec* types and their impact in clinical outcome should be addressed in future studies. Failure to type the SCC*mec* carried by four isolates all clonally related, probably results from the typing protocol used, as this only detects eight SCC*mec* types and subtypes (Zhang *et al.*, 2005) out of the fourteen SCC*mec* types known to date (Yamaguchi *et al.*, 2020). As with our findings, the original protocol of SCC*mec* typing also reported non typeable strains suggesting the presence of new types or variant sub-types as result of new structural types and subtypes or structural rearrangements and recombination of the *mec* element (Zhang *et al.*, 2005). Therefore, before we presume the occurrence of new types or variant sub-types, the non-typable SCC*mec* from our study (Chapter 3) should be sequenced or reanalyzed by additional protocols screening for additional SCC*mec* types and sub-types known to date.

Apart from MRSA strains, for which the *mecA* gene confers resistance to penicillin and other

β-lactam antibiotics**,** penicillin resistance was mainly encoded by *blaZ* gene, often carried in plasmids (Haaber *et al.*, 2017). Noteworthy, two strains presented a discordant phenotypic and genotypic background to penicillin (Chapter 3). The first one (belonging to CC152) was resistant to penicillin (and also to erythromycin and clindamycin) but with negative results for *blaZ*, probably as the result of mutation(s) in the primer-annealing sites, thus preventing amplification. The second strain (belonging to CC5) carried *blaZ* but showed a pan-susceptible phenotype, eventually from absent gene expression related to mutation in the coding region. These hypotheses should be confirmed by additional analyses (DNA sequencing, gene expression assays).

Regarding tetracycline resistance, it was mostly associated to the plasmid-borne genes *tet*(K) and *tet*(L), linked to tetracycline efflux, and to *tet*(M), involved in ribosomal protection. Co-trimoxazole resistance was mainly linked to the transposon-encoded *dfrG* and *dfrA*(S1) genes. The observed resistance to clindamycin, an antibiotic unavailable in Mozambique, was related to the plasmid located *erm*(C) and *msr*(A) genes. These genes are also associated to resistance to erythromycin, which may explain the resistance observed to clindamycin through primary use of erythromycin in our setting. Low resistance rate to gentamicin and chloramphenicol were associated with the determinants *aacA-aphD* (transposon located) and cat_{pC221} (plasmid or transposon located), respectively. For a few isolates, we detected discrepancies between the resistance phenotype and carriage of a resistance determinant, which could be related to the presence of other genes not screened in this study, namely (*tet*(X) or *tet*(O) (encoding resistance to tetracycline), *dfrB* or *dfrK* (co-trimoxazole resistance), *fexA* or /*cfr* (resistance to chloramphenicol) or *erm*(B) (erythromycin resistance). This analysis revealed that most antimicrobial resistance phenotypes detected in our study collection are related to mobile genetic elements, such as plasmids, transposons and gene cassettes. Therefore, future investigation of our *S. aureus* collection must address the detailed characterization of the mobile genomic elements carrying resistance determinants described and virulence factors (such as bacteriophages encoding PVL toxin) (Chapter 4), as these elements often carry additional resistance determinants and virulence factors that can be easily transferred between strains (Haaber *et al.*, 2017; Partridge *et al.*, 2018).

5.5. SAB-related *S. aureus* clonal lineages circulating in the Manhiça District

Bacterial phenotype and genotype have been described as highly predictive factors for adverse infection outcome (Horváth *et al.*, 2020; Recker *et al.*, 2017). Noteworthy, we observed the highest mortality rates (36%) among children infected with strains from CC8 (Chapter 3); a worldwide disseminated *S. aureus* clone (Strauß *et al.*, 2017) frequently associated with outbreaks (Lee *et al.*, 2018). In fact, the majority (15 out of 16) MRSA strains found in our study were from CC8 (mostly ST612 and ST8) (Chapter 3), the latter from the same lineage of the hypervirulent clone USA300 (Nimmo, 2012; Strauß *et al.*, 2017). These findings revealed that despite the low circulation of CC8 in our setting in the last years of the surveillance, this lineage should be monitored closely. The lineage ST612 from our study is frequently reported in neighboring countries that share borders with Mozambique (South Africa and Tanzania) (Jansen van Rensburg *et al.*, 2011; Lawal *et al.*, 2022; Moodley *et al.*, 2010; Moremi *et al.*, 2019; Oosthuysen *et al.*, 2014; Perovic *et al.*, 2015) and Australia (Axon *et al.*, 2011; Groves *et al.*, 2016), and also associated to veterinary practices. There are still few studies that characterized *S. aureus* circulating in animals in our setting. We recently reported the analysis of *S. aureus* isolated from raw cow´s milk in Manhiça, revealing the circulation of human adapted strains with predominance of ST1/CC1, ST152/CC152 and ST8/CC8 (Nhatsave *et al.*, 2021). Interestingly, the CC152 lineage isolated from animal milk in 2019 (Nhatsave *et al.*, 2021), is the same emergent (CC152), lineage predominant in the last years of surveillance among children with SAB in our setting (Chapter 3). These data suggest the need of additional studies to understand the dynamic transmission of *S. aureus* strains between animals and humans in close contact in the Manhiça district.

Previous studies reported age and comorbidities as predictive factors of mortality among patients infected with *S. aureus* from CC22 (Recker *et al.*, 2017), however the contribution of comorbidities on clinical condition of the patients was not analysed in detail in our study. Despite no association observed with infection by CC2 strains and mortality in the present study, this clonal lineage was associated with extended LOS (Chapter 3) perhaps due to severe clinical disease. Lineage CC2 is one of the most common healthcare-associated MRSA lineage in Europe (Toleman *et al.*, 2017), and its detection (including one strain ciprofloxacin resistant carrying QRDR mutations) in Manhiça district in distinct years (in

2006, 2009, 2011, 2016 and 2017) is worrisome.

An interesting finding regarding the epidemiology of *S. aureus* strains in our setting was the decrease of specific clonal lineages (e.g. CC1, CC5, CC8, CC15, CC25, CC80, CC88) over the study period and the emergence of CC152 in the last years (2010 onwards). The factors behind these clonal alterations remain to be clarified, although some reports suggest competition between lineages (Lakhundi and Zhang, 2018; Ruffing *et al.*, 2017). The MRSA-CC152 has been identified in African countries that had not previously reporting its circulation, including the Democratic Republic of Congo, Kenya, Nigeria and South Africa (Lawal *et al.*, 2022). Thus, the role of these important local and regional clones (CC152-MSSA and CC152-MRSA, respectively) needs more detailed studies and surveillance. Noteworthy, the epidemiology of CC152-MRSA mimics in multiple ways the one of CC80-MRSA, through the emergence from a PVL-positive CC80-MSSA ancestor from North Africa/Europe (Baig *et al.*, 2020). The well-known "African clone" (ST88) presenting methicillin resistance is homogenously distributed across Africa (Lawal *et al.*, 2022; Schaumburg *et al.*, 2014). In contrast, in our study all but one of ST88 strains were methicillin susceptible, suggesting distinct geographical distribution of important lineages in African context. Interestingly, this clone was not detected in our setting since 2015, nor was it associated to poor outcome among infected patients (Chapter 3).

In our study, specific *S. aureus* clones presented particular resistance phenotypes, namely CC25 showing resistance to co-trimoxazole, CC8-MRSA showing combined resistance to gentamicin, chloramphenicol and co-trimoxazole, and CC22 with resistance to gentamicin (Chapter 3). These observations suggest that the presence of specific clonal lineages can be early suspected, based on phenotypic resistance results, allowing prompt management of patients presumably infected by clonal lineage of concern in our setting.

We also observed that particular *S. aureus* lineages were found exclusively in some age strata, while others were scattered among distinct ones (Chapter 3), suggesting that some clones are age strata adapted, as already described (Sangvik *et al.*, 2011). The interaction between host susceptibility and infection by specific clonal lineages of *S. aureu*s should be addressed in future studies.

5.6. Virulence potential of *S. aureus* clones associated to SAB in the Manhiça district

The emergence of CC121 and CC152 MSSA lineages in the last years of surveillance in our setting mirrors the epidemiological landscape in several sub-Saharan African countries. These clonal lienages are particularly worrisome as they are often associated with potentially increased virulence (Ackers *et al.*, 2021; Rao *et al.*, 2015). Thus, we evaluated the virulence traits associated with these relevant lineages. Corroborating previous studies, PVL carriage was a highly frequent characteristic of CC152 and CC121 strains. Additionally, CC152 strains showed a remarkable ability to form biofilms. Despite the high prevalence of PVL observed in our collection, this toxin was not associated with extended LOS nor increased mortality in patients with SAB. The contribution of this toxin to invasive infections, such as bacteraemia is still controverse (McGuire *et al.*, 2023). Similarly to PVL, production of biofilm was not associated with poor clinical outcomes. These data suggest the role of additional virulence factors in the pathogenesis of SAB in our setting.

The *in vivo* analysis in the *G. mellonella* infection model revealed that the CC121 and CC152 strains were more virulent when compared to a reference strain, suggesting the virulence potential of these CCs circulating in the last years of surveillance. The data collected also revealed a similar virulence potential between the emergent lineages and CC8, a lineage decreasingly detected in our setting during the surveillance timeframe. However, this exploratory study (Chapter 4) only provided a snapshot of a small collection of emergent clones circulating in Manhiça district. Future studies in our setting may explore *G. mellonella* infection model to evaluate the virulence profile of larger collections of *S. aureus* and other relevant bacterial pathogens associated with bacteraemia in Manhiça district (e.g. *K. pneumoniae*, *S. pneumoniae*), as well as to evaluate the efficacy of alternative/novel antimicrobial drugs against the circulating MDR strains.

The *G. mellonella* innate immune response has remarkable similarities with mammalian models, allowing a correlation between both models (Tsai *et al.*, 2016). Similar to *G. mellonella* (Phylum Arthropoda, class Lepidoptera) infection model, there are other invertebrate models far applied to elucidate the virulence of *S. aureus*, such as the *Drosophila melanogaster* (Phylum Arthropoda, class Insecta) and the *Caenorhabditis elegans* (Phylum Nematoda, class Chomadorea). A good correlation has been described between *G. mellonella*

and those models. When compared to other *D. melanogaster* and *C. elegans* models, *G. mellonella* has several advantages, as this invertebrate is easily manipulated due to its larger size (3-30 mm compared to 3 and 1 mm for *D. melanogaster* and *C. elegans*, respectively), and its adaptive behavior to survive at 37 °C, the normal temperature of the human body and optimal for the growth of majority bacterial pathogens, allow to study the expression for many virulence genes (Serrano *et al.*, 2023). In addition, the innate immune system of *G. mellonella* is more developed compared to the *C. elegans*, allowing microorganisms phagocytosis, a characteristic absent in *C. elegans*. In contrast to *D. melanogaster*, the assays for *G. mellonella* do not require specialized experience and equipment. Those characteristics confers advantages to *G. mellonellla* for *in vivo* studies for distinct pathogens (bacteria and fungi), as well as for assessing the efficacy of novel antimicrobial drugs, before proceeding to preclinical studies in mammals (Desbois and Coote, 2011). Despite the advantages of the use of *G. mellonella* and other invertebrates as *in vivo* infection models, their use is inadequate to understand the adaptive immune system response (Ménard *et al.*, 2021).
The *G. melllonella* model also offers several advantages over mammalian models, as it does not require ethical approval, is easily manipulated, is inexpensive, allows large-scale breeding, and allows a rapid experimental execution (Ménard *et al.*, 2021; Tsai *et al.*, 2016). Therefore, the *G. mellonella* infection model is suitable to implementation in tropical regions and low-income countries countries with limited resources, including those from sub-Saharan Africa. Transferring this technique to Manhiça (Mozambique) will be helpful in investigating the virulence potential of important bacterial and fungal pathogens of interest. CISM has experience in working with invertebrates, as we have successful implemented the breeding of colonies of *Anopheles* mosquitoes for insecticide resistance assays. In addition, CISM is implementing next-generation sequencing technologies and cell culture assays, and the introduction of infection models will further strengthen our research facilities.

Overall, this is one of the first studies evaluating in detail the epidemiology of SAB and the antimicrobial susceptibility, clonality and virulence of SAB-related *S. aureus* strains in Mozambique. Similar study designs must be extended to other regions in Mozambique to determine the national burden of SAB and the molecular and phenotypic pattern of

circulating *S. aureus* for better defining and implementing of control and prevention measures. This can be complemented by designing more comprehensive studies to understand the transmission dynamics of *S. aureus* in One Health approach.

This thesis contributed to filling the gap in terms of scientific critical mass and technical knowledge for the young generation of researchers in Mozambique. This gain is not only for Mozambique as a country but also for other countries within the PALOP community, as this network of Portuguese-speaking countries has established several exchange programs as capacity-building strategies.

5.7. Main Conclusions

This doctoral study presents important epidemiological and molecular information not previously available on *S. aureus* bacteraemia affecting children in southern Mozambique. Despite the declining rate of SAB in our population, the mortality rate is still high, possibly related to infection by MDR and MRSA strains untreatable with the commonly available antibiotics. These findings suggest an urgent need to review the current guidelines of empiric antibiotic treatment in Mozambique, as supported by the current concern regarding the burden of antimicrobial resistance.

Specific clonal lineages were associated with poor clinical outcome, and the emergence of important *S. aureus* lineages (CC121 and CC152) carrying *lukSF-PV* genes toxins was reported. Strains from the lineage CC152 and, in a lesser extent, CC121, are frequent strong biofilm producers and show higher virulence potential in the *G. mellonella* infection model when compared to a prototype reference strain suggesting the need of continuous surveillance, as those strains may impact negatively the management of paediatric SAB in Manhiça, Mozambique.

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6. Annexes

6.1 Supplementary Tables for Chapter 3

Antimicrobial resistance and clonality of *Staphylococcus aureus* causing bacteraemia in children admitted to the Manhiça District Hospital, Mozambique, over two decades

Table S3.1. Primers used in this study.

Target Gene	Primers	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Reference
		***S. aureus* identification**		
nuc	nuc_Fw	GCGATTGATGGTGATACGGTT	270	(Brakstad *et al.*, 1992)
	nuc_Rv	AGCCAAGCCTTGACGAACTAAAGC		
		Screening of resistance determinants		
mecA	mecA_Fw	GGTCCCATTAACTCTGAAG	1040	(Petinaki *et al.*, 2001)
	mecA_Rv	AGTTCTGCAGTACCGGATTTGC		
blaZ	blaZ_Fw	GATAAGAGATTTGCCTATGC	533	(Milheiriço *et al.*, 2011)
	blaZ_Rv	GCATATGTTATTGCTTGACC		
erm(A)	erm(A)_Fw	AAGCGGTAAACCCCTCTGAG	442	(Jensen *et al.*, 2002)
	erm(A)_Rv	AAGCGGTAAACCCCTCTGAG		
erm(C)	erm(C)_Fw	TCGTAACTGCCATTGAAATA	348	(Costa *et al.*, 2016)
	erm(C)_Rv	TCACTTTAGGTTTAGGATGAAA		
msr(A)	msr(A)_Fw	GATTGTCCCAAGCCAGTAAA	445	(Ferreira *et al.*, 2021)
	msr(A)_Rv	GCCATTTGCACTTTAGGAGA		
tet(K)	tet(K)_Fw	GTAGCGACAATAGGTAATAGT	361	(Strommenger *et al.*, 2003)
	tet(K)_Rv	GTAGTGACAATAAACCTCCTA		
tet(M)	tet(M)_Fw	GTTAAATAGTGTTCTTGGAG	657	(Aarestrup *et al.*, 2000)
	tet(M)_Rv	CTAAGATATGGCTCTAACAA		
tet(L)	tet(L)_Fw	GTCGGTAATTGGGTTTGTTG	421	(Costa *et al.*, 2021)
	tet(L)_Rv	TGACAGCACGCTAACGATAA		
aacA-aphD	aacA-aphD_Fw	CAGAGCCTTGGGAAGATGAAG	348	(Vakulenko *et al.*, 2003)
	aacA-aphD_Rv	CCTCGTGTAATTCATGTTCTGGC		
dfrG	dfrG_Fw	TTTCTTTGATTGCTGCGATG	501	(Couto *et al.*, 2014)
	dfrG_Rv	AACGCACCCGTTAACTCAAT		
dfrA(S1)	dfrA(S1)_Fw	CACTTGTAATGGCACGGAAA	270	(Argudín *et al.*, 2011)
	dfrA(S1)_Rv	CGAATGTGTATGGTGGAAAG		
grlA	grlA_Fw	CAAGAGCGTGCTTTRCCT	300	(Costa *et al.*, 2021)
	grlA_Rv	CTGACTYAATTTCGCTTCAG		
gyrA	gyrA_Fw	ATGAGTGTTATYGTRTCTCGT	261	
	gyrA_Rv	CATMGAACCRAAGTTACCTTG		
$catp_{C221}$	catpC221_Fw	ATTTATGCAATTATGGAAGTTG	435	(Schnellmann *et al.*, 2006)
	catpC221_Rv	TGAAGCATGGTAACCATCAC		

bp: base pair; **Fw:** "forward"; **Rv: "**reverse"; R: A + G; Y: C + T; M: A + C.

Table S3.1. ***(Cont.)*** **Primers used in this study.**

Target Gene	Primers	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Reference
		***spa* typing**		
spa	spa-1113-Fw	TAAAGACGATCCTTCGGTGAGC	110-442	(Aires-de-Sousa *et al.*, 2006)
	spa-1514-Rv	CAGCAGTAGTGCCGTTTGCTT		
		MLST		
arcC	arcC_Fw	CCT TTATTTGATTCACCAGCG	456	(Crisostomo *et al.*, 2001) (Enright *et al.*, 2000)
	arcC_Rv	AGGTATCTGCTTCAATCAGCG		
aroE	aroE_Fw	ATCGGAAATCCTATTTCACATTC	456	(Enright *et al.*, 2000)
	aroE_Rv	GGTGTTGTATTAATAACGATATC		
glpF	glpF_Fw	CTAGGAACTGCAATCTTAATCC	465	(Enright *et al.*, 2000)
	glpF_Rv	TGGTAAAATCGCATGTCCAATTC		
gmk	gmk_Fw	ATCGTTTTATCGGGACCATC	429	(Enright *et al.*, 2000)
	gmk_Rv	TCATTAACTACAACGTAATCGTA		
pta	pta_Fw	GTTAAAATCGTATTACCTGAAGG	474	(Enright *et al.*, 2000)
	pta_Rv	GACCCTTTTGTTGAAAAGCTTAA		
tpi	tpi_Fw	TCGTTCATTCTGAACGTCGTGAA	402	(Enright *et al.*, 2000)
	tpi_Rv	TTTGCACCTTCTAACAATTGTAC		
yqiL	yqiL_Fw	CAGCATACAGGACACCTATTGGC	516	(Enright *et al.*, 2000)
	yqiL_Rv	CGTTGAGGAATCGATACTGGAAC		
		SCC*mec* typing		
SCC*mec*I	SCCmecI_Fw	GCTTTAAAGAGTGTCGTTACAGG	613	(Zhang *et al.*, 2005)
	SCCmecI_Rv	GTTCTCTCATAGTATGACGTCC		
SCC*mec*II	SCCmecII_Fw	CGTTGAAGATGATGAAGCG	398	(Zhang *et al.*, 2005)
	SCCmecII_Rv	CGAAATCAATGGTTAATGGACC		
SCC*mec*III	SCCmecIII_Fw	CCATATTGTGTACGATGCG	280	(Zhang *et al.*, 2005)
	SCCmecIII_Rv	CCTTAGTTGTCGTAACAGATCG		
SCC*mec*IVa	SCCmecIVa_Fw	GCCTTATTCGAAGAAACCG	776	(Zhang *et al.*, 2005)
	SCCmecIVa_Rv	CTACTCTTCTGAAAAGCGTCG		
SCC*mec*IVb	SCCmecIVb_Fw	TCTGGAATTACTTCAGCTGC	493	(Zhang *et al.*, 2005)
	SCCmecIVb_Rv	AAACAATATTGCTCTCCCTC		
SCC*mec*IVc	SCCmecIVc_Fw	ACAATATTTGTATTATCGGAGAGC	200	(Zhang *et al.*, 2005)
	SCCmecIVc_Rv	TTGGTATGAGGTATTGCTGG		
SCC*mec*IVd	SCCmecIVd_Fw	CTCAAAATACGGACCCCAATACA	881	(Zhang *et al.*, 2005)
	SCCmecIVd_Rv	TGCTCCAGTAATTGCTAAAG		
SCC*mec*V	SCCmecV_Fw	GAACATTGTTACTTAAATGAGCG	325	(Zhang *et al.*, 2005)
	SCCmecV_Rv	TGAAAGTTGTACCCTTGACACC		

bp: base pair; **Fw:** "forward"; **Rv: "**reverse"; R: A + G; Y: C + T; M: A + C.

Table S3.2. Results from previous studies showing similarity or agreement between results from *spa* typing and MLST of *S. aureus.*

#	*spa* type	Sequence type	Clonal complex	References
1	t008	ST8	CC8	(Silva *et al.*, 2022)
				(Goudarzi *et al.*, 2020)
				(Wang *et al.*, 2022)
		ST8 (+++)/ST247(+)		(Asadollahi *et al.*, 2018)
		ST8/ST113		(Boswihi *et al.*, 2016)
		ST8		(Goudarzi *et al.*, 2021)
2	t064	ST8	CC8	(Khan *et al.*, 2021)
				(Goudarzi *et al.*, 2019)
				(Boswihi *et al.*, 2016)
				(Goudarzi *et al.*, 2020)
3	t084	ST15 (+++)	CC15	(Creutz *et al.*, 2022)
				(Asadollahi *et al.*, 2018)
				(Goudarzi *et al.*, 2019)
				(Sahin-Tóth *et al.*, 2021)
		ST1535 (+)		(Udo *et al.*, 2020)
4	t002	ST5	CC5	(Goudarzi *et al.*, 2019)
				(Boswihi *et al.*, 2016)
				(Asadollahi *et al.*, 2018)
5	t701	ST6	CC5	(He *et al.*, 2021)
				(Liao *et al.*, 2020)
6	t127	ST1	CC1	(Huang *et al.*, 2021)
				(He *et al.*, 2021)
				(Hait *et al.*, 2021)
				(Boudet *et al.*, 2021)
				(Boswihi *et al.*, 2016)
				(Petersen *et al.*, 2021)
7	t1299	ST152	CC152	(Nhatsave *et al.*, 2021)
8	t148	ST72	CC8	(Tavares *et al.*, 2014)
				(Garcia *et al.*, 2016)
9	t186	ST78	CC88	(Earls *et al.*, 2018)
		ST88		(Ohadian Moghadam *et al.*, 2017)
				(Kpeli *et al.*, 2016)
10	t355	ST152	CC152	(Kpeli *et al.*, 2016)
				(Egyir *et al.*, 2021)
				(Egyir *et al.*, 2020)
				(Nyasinga *et al.*, 2019)
11	t645	ST121	CC121	(Haque *et al.*, 2019)
12	t010	ST149	CC5	(Mairi *et al.*, 2021)
		ST5		(Rijnders *et al.*, 2009)
		ST2633		(Li *et al.*, 2019)
13	t174	ST1	CC1	(Parisi *et al.*, 2016)
				(Normanno *et al.*, 2015)
14	t376	ST80	CC80	(Mairi *et al.*, 2020)
				(Boswihi *et al.*, 2016)

(+++): Most common sequence type (ST) identified among specific *spa* type; **(+)** less predominant ST among specific *spa* type.

Table S3.2. ***(Cont.)*** **Results from previous studies showing similarity or agreement between results from *spa* typing and MLST of *S. aureus.***

#	*spa* type	Sequence type	Clonal complex	References
15	t015	ST45	CC45	(Kwapisz *et al.*, 2020)
		not identified (microarray typing)		(Morach *et al.*, 2019)
		ST45		(Loncaric *et al.*, 2019)
		not identified (microarray typing)		(Deasy *et al.*, 2019)
16	t1198	ST80 (SLV of ST6994)	CC80	(Islam *et al.*, 2019)
				(Aung *et al.*, 2017)
17	t2793	not identified (microarray typing)	CC45	(Vubil *et al.*, 2017)
			CC121	
18	t317	ST121	CC121	(Breurec *et al.*, 2011)
19	t888	ST12		(Nethercott *et al.*, 2013)
		not determined		(Garbacz *et al.*, 2021)
20	t891	ST22	CC22	(Antiabong *et al.*, 2017)
21	t1476	ST8	CC8	(Lebughe *et al.*, 2017)
				(Schaumburg *et al.*, 2015)
				(Vandendriessche *et al.*, 2017)
22	t3772	ST25	CC25	(Schaumburg *et al.*, 2011)
		not identified (microarray typing)		(Vubil *et al.*, 2017)

(+++): Most common sequence type (ST) identified among specific *spa* type; **(+)** less predominant ST among specific *spa* type.

Table S3.3. Resistance profiles among multidrug resistant (MDR) and methicillin resistant (MRSA) *S. aureus* (N = 86).

Resistance patterns profile	MDR % (n)	MRSA % (n)
FOX-TCY-PEN	0	1 (1)
ERY-CLID-TCY	1 (1)	0
CHL-ERY-CLID-PEN	1 (1)	0
CHL-ERY-CLID-TCY-PEN	1 (1)	0
CHL-TCY-PEN	1 (1)	0
ERY-TCY-PEN	1 (1)	0
GEN-SXT-CIP-PEN	1 (1)	0
SXT-CHL-ERY-CLID-TCY-PEN	1 (1)	0
SXT-CHL-TCY-PEN	1 (1)	0
FOX-ERY-CLID-PEN	1 (1)	1 (1)
FOX-ERY-CLID-TCY-PEN	1 (1)	1 (1)
GEN-SXT-FOX-ERY-CLID-TCY-PEN	1 (1)	1 (1)
SXT-CHL-FOX-ERY-CLID-TCY-PEN	1 (1)	1 (1)
GEN-FOX-ERY-CLID-TCY-PEN	2 (2)	2 (2)
SXT-ERY-CLID-PEN	7 (6)	0
SXT-ERY-CLID-TCY-PEN	7 (6)	0
SXT-TCY-PEN	8 (7)	0
GEN-SXT-CHL-FOX-ERY-CLID-TCY-PEN	10 (9)	10 (9)
ERY-CLID-PEN	20 (17)	0
ERY-CLID-TCY-PEN	30 (26)	0

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin.

Table S3.4. Frequency and level of agreement between antibiotic susceptibility testing (AST) and its resistance determinants.

Penicillin (PEN)

Antibiotic susceptibility testing	Resistance determinant: *blaZ*		Total
	Negative	Positive	
Susceptible	31	1	32
Resistant	1	303	304
Total	32	304	336
Agreement (%)	99.40%		
Kappa coefficient	0.965	95% CI: 0.918 to 1.000	

Cefoxitin (FOX)

Antibiotic susceptibility testing	Resistance determinant: *mecA*		Total
	Negative	Positive	
Susceptible	320	0	320
Resistant	0	16	16
Total	320	16	336
Agreement (%)	100%		
Kappa coefficient	1.000	95% CI: 1.000 to 1.000	

Tetracycline (TCY)

Antibiotic susceptibility testing	Resistance determinant: *tet(*K)		Total
	Negative	Positive	
Susceptible	166	9	175
Resistant	39	122	161
Total	205	131	366
Agreement (%)	85.71%		
Kappa coefficient	0.716	95% CI: 0.637 to 0.786	

Table S3.5. Phenotypic resistance and resistance determinants among all *S. aureus* clonal complexes detected in this study.

CC	Resistance patterns (n)	Main resistance determinants (n)
CC5 (N=58, MRSA: 0; MDR: 6 (10%))	PEN (31)	*blaZ* (31)
	PEN-TCY (14)	*blaZ-tet*(K) (12); *blaZ-tet*(K)-*tet*(L)-*tet*(M) (1); *blaZ-tet*(L)-*tet*(M) (1)
	PEN-TCY-ERY-CLID (2)	*blaZ-tet*(K)-*erm*(C) (1); *blaZ-tet*(L)-*tet*(M)-*msr*(A) (1)
	PEN-ERY-CLID (2)	*blaZ* (1); *blaZ-erm*(C) (1)
	PEN-SXT-ERY-CLID (1)	*blaZ-tet*(K)-*dfrG* (1)
	TCY-ERY-CLID (1)	*tet*(K) (1)
	PEN-ERY (1)	*blaZ-erm*(C) (1)
	ERY-CLID (1)	*erm*(C) (1)
	Fully susceptible (5)	*blaZ* (1)
CC8 (N=56, MRSA: 15 (27%); MDR: 22 (39%))	PEN (15)	*blaZ* (14); *blaZ-tet*(K) (1)
	PEN-TCY (16)	*blaZ-tet*(K) (13); *blaZ-tet*(K)-*tet*(L) (1); *blaZ-tet*(L)-*tet*(M) (1); *blaZ-tet*(L) (1)
	FOX-PEN-TCY-GEN-SXT-CHL-ERY-CLID (9)	*blaZ-tet*(M)-*mecA*-*erm*(C)-*aacA_aphD-dfrA*(S1)-*cat* (5); *blaZ-tet*(K)-*tet*(M)-*mecA*-*erm*(C)-*msrA-aacA_aphD-dfrA*(S1)-*dfrG-cat* (1); *blaZ-tet*(L)-*tet*(M)-*mecA*-*erm*(C)-*aacA_aphD-dfrA*(S1) (1); *blaZ-tetM-mecA-ermC-aacA_aphD-dfrA*(S1) (1); *blaZ-tet*(K)-*tet*(M)-*mecA*-*erm*(C)-*aacA_aphD-dfrA*(S1) (1)
	PEN-TCY-ERY-CLID (5)	*blaZ-tet*(L)-*tet*(M)-*erm*(C) (3); *blaZ-tet*(K)-*erm*(C) (1); *blaZ* (1)
	FOX-PEN-TCY-GEN-ERY-CLID (2)	*blaZ-tet*(K)-*tet*(L)-*tet*(M)-*mecA*-*erm*(C)-*aacA_aphD* (1); *blaZ-tet*(L)-*tet*(M)-*mecA*-*erm*(C)-*aacA_aphD* (1)
	FOX-PEN-TCY-GEN-SXT-ERY-CLID (1)	*blaZ-tet*(K)-*tet*(M)-*mecA*-*erm*(C)-*msrA-aacA_aphD-dfrA*(S1) (1)
	FOX-PEN-TCY-SXT-CHL-ERY-CLID (1)	*blaZ-tet*(M)-*mecA*-*erm*(C)-*dfrA*(S1)-*cat* (1)
	FOX-PEN-TCY-ERY-CLID (1)	*mecA-blaZ-tet*(L)-*tet*(M)-*erm*(C) (1)
	FOX-PEN-TCY (1)	*mecA-blaZ-tet*(K)-*tet*(L)-*tet*(M) (1)
	PEN-TCY-SXT (2)	*blaZ-tet*(K)-*dfrG* (2)
	PEN-ERY (1)	*blaZ* (1)
	PEN-TCY-CHL-ERY-CLID (1)	*blaZ-tet*(L)-*erm*(C) (1)
	PEN-CHL (1)	*blaZ* (1)

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLID, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin; CIP, ciprofloxacin.

Table S3.5. ***(Cont.)*** **Phenotypic resistance and resistance determinants among all** ***S. aureus*** **clonal complexes detected in this study.**

CC	Resistance patterns (n)	Main resistance determinants (n)
CC15 (N=37, MRSA:0, MDR: 2 (5%))	PEN (18)	*blaZ* (16); *blaZ-tet*(K) (1); *blaZ-tet*(K)-*tet*(L) (1)
	PEN-TCY (17)	*blaZ-tet*(K) (16); *blaZ-tet*(K)-*tet*(L) (1)
	ERY-CLID-TCY-PEN (2)	*blaZ-tet*(K)-*erm*(C) (2)
CC1 (N=36, MRSA: 0; MDR: 9 (25%))	PEN (14)	*blaZ* (12); *blaZ-tetK* (2)
	PEN-TCY (7)	*blaZ-tet*(K) (5); *blaZ-tet*(K)-*tet*(L) (1); *blaZ* (1)
	PEN-TCY-ERY-CLID (3)	*blaZ-tet*(K)-*erm*(C)-*msr*(A) (1); *blaZ-tet*(K)-*erm*(C) (1); *blaZ-tet*(L)- *tet*(M) (1)
	PEN-ERY-CLID (3)	*blaZ-erm*(C) (3)
	PEN-TCY-SXT-ERY-	*blaZ-tet*(K)-*erm*(C) (1)
	PEN-TCY-SXT (1)	*blaZ-tet*(K)-*dfrG* (1)
	PEN-TCY-CHL (1)	*blaZ-tet*(K)-*cat* (1)
	PEN-ERY (1)	*blaZ* (1)
	PEN-CLID (1)	*blaZ* (1)
	TCY (2)	*tet*(M) (1)
	Fully susceptible (2)	
CC121 (N=34, MRSA: 0; MDR: 9 (26%))	PEN-TCY (11)	*blaZ-tet*(M) (7); *blaZ-tet*(K) (2); *blaZ-tet*(K)-*tet*(L) (1);
	PEN (10)	*blaZ* (10)
	PEN-TCY-ERY-CLID (6)	*blaZ-tet*(K)-*erm*(C) (6)
	PEN-ERY (3)	*blaZ-erm*(C) (2); *blaZ* (1)
	PEN-SXT-ERY-CLID (1)	*blaZ- dfrG-ermC* (1)
	PEN-SXT-TCY (1)	*blaZ- dfrG-tetM* (1)
	PEN-ERY-CLID (1)	*blaZ-erm(C)* (1)
	Fully susceptible (1)	
CC152 (N=33, MRSA: 0; MDR: 12 (36%))	PEN (14)	*blaZ* (14)
	PEN-ERY-CLID (8)	*blaZ* (3); *blaZ-erm*(C) (3); *blaZ-tet*(K)-*erm*(C)-*msr*(A) (1); *blaZ-tet*(K)-*erm*(C) (1)
	PEN-TCY (5)	*blaZ-tet*(K)-*tet*(L) (3); *blaZ-tet*(M)-*tet*(L) (1); *blaZ-*
	PEN-TCY-ERY-CLID (4)	*blaZ-tet*(K)-*tet*(L)-*erm*(C) (1); *blaZ-tet*(K)-*erm*(C) (1); *blaZ-tet*(M)-*erm*(C) (1); *blaZ-erm*(C) (1)
	Fully susceptible (2)	
CC88 (N=26, MRSA: 1 (4%); MDR: 6 (23%))	PEN-TCY (15)	*blaZ-tet*(K) (14); *blaZ-tet*(L)-*tet*(M) (1)
	PEN (2)	*blaZ* (2)
	PEN-ERY (2)	*blaZ* (2)
	PEN-TCY-ERY-CLID (2)	*blaZ-tet*(L)-*tet*(M)-*erm*(C) (1); *blaZ-tet*(K) (1)
	FOX-PEN-ERY-CLID (1)	*blaZ-mecA-erm*(C) (1)
	PEN-SXT-ERY-CLID (1)	*blaZ-erm*(C) (1)
	PEN-TCY-ERY (1)	*blaZ-tet*(K) (1)
	PEN-ERY-CLID (1)	*blaZ-erm*(C) (1)
	PEN-SXT (1)	*blaZ* (1)

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLID, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin; CIP, ciprofloxacin.

Table S3.5. ***(Cont.)*** **Phenotypic resistance and resistance determinants among all** ***S. aureus*** **clonal complexes detected in this study.**

CC	Resistance patterns (n)	Main resistance determinants (n)
CC25 (N=20, MRSA: 0; MDR: 16 (80%))	PEN-TCY-SXT-ERY-CLID (5)	*blaZ-tet*(K)-*erm*(C)-*dfrG* (5)
	PEN-SXT-ERY-CLID (3)	*blaZ-erm(C)-dfrG* (); *blaZ-erm(C)* (1)
	PEN-TCY-SXT (3)	*blaZ-tet*(K)-*dfrG* (3)
	PEN-SXT (2)	*blaZ-dfrG* (1); *blaZ* (1)
	PEN-TCY-SXT-CHL-ERY-CLID (1)	*blaZ-tet*(K)-*erm*(C)-*dfrG* (1)
	PEN-TCY-SXT-CHL (1)	*blaZ-tet*(K)-*dfrG-cat* (1)
	PEN-TCY-ERY-CLID (1)	*blaZ-tetK-erm*(C) (1)
	PEN-CHL-ERY-CLID (1)	*blaZ-erm(C)-cat* (1)
	PEN-ERY-CLID (1)	*blaZ-msrA* (1)
	PEN-TCY (1)	*blaZ-tet*(K) (1)
	PEN (1)	*blaZ* (1)
CC80 (N=16, MRSA: 0; MDR: 1 (6%))	TCY (7)	*tet*(K) (7)
	PEN-ERY-CLID (1)	*blaZ-erm*(C) (1)
	PEN-TCY (1)	*blaZ-tet*(K) (1)
	Fully susceptible (7)	
CC45 (N=8, MRSA: 0, MDR: 1 (13%))	PEN (4)	*blaZ* (4)
	PEN-TCY (2)	*blaZ-tet*(K) (2)
	PEN-TCY-ERY-CLID (1)	*blaZ-tet*(K)-*erm*(C) (1)
	Fully susceptible (1)	
CC22 (N=6, MRSA: 0, MDR: 1 (17%))	PEN (3)	*blaZ* (3)
	PEN-GEN (2)	*blaZ-aacA_aphD* (2)
	PEN-GEN-SXT-CIP (1)	*blaZ-dfrA(S1)* (1)
CC12 (N=3, MRSA: 0, MDR: 3 (100%))	PEN (2)	*blaZ* (1); *blaZ-tet*(K) (1)
	TCY (1)	*tet*(K) (1)
Singleton (N=2, MRSA: 0, MDR: 0)	PEN-TCY (2)	*blaZ-tet*(K) (2)
	TCY-ERY (1)	*tet(*K) (1)
CC97 (N=1, MRSA: 0, MDR: 0)	Fully susceptible (1)	

PEN, penicillin; FOX, cefoxitin; TCY, tetracycline; ERY, erythromycin; CLID, clindamycin; SXT, co-trimoxazole; CHL, chloramphenicol; GEN, gentamicin; CIP, ciprofloxacin.

References for Supplementary Tables of Chapter 3

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6.2 Supplementary Figure for Chapter 4

Exploring the virulence potential of CC152 and CC121 *S. aureus* strains related to paediatric community-acquired bacteraemia in the Manhiça District Hospital, Mozambique

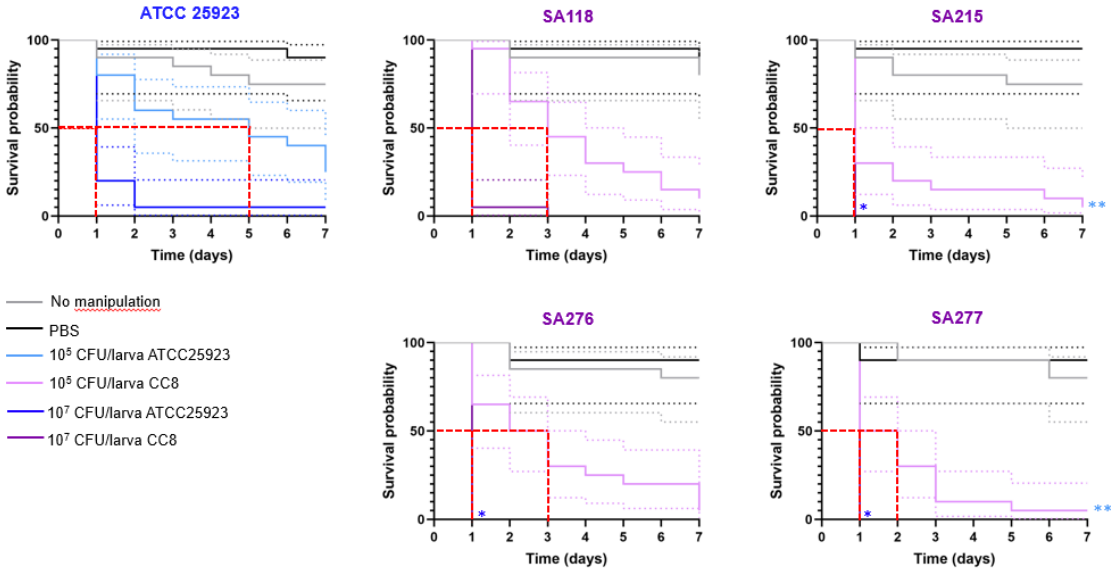

Figure S4.1. Kaplan-Meier survival analysis of *G. mellonella* infection assays with *S. aureus* ATCC25923 (in blue) and SAB-related *S. aureus* strains representative of clonal lineage CC8 (in purple). The dotted lines indicate the 95% confidence intervals and the red lines indicate the median survival time. Statistically significant differences between each strain and *S. aureus* ATCC25923 (at corresponding inoculums) are identified as follows: * $p < 0.05$ and ** $p < 0.01$.