Epidemiological and Phylogenetic Investigation of Panton-Valentine Leukocidin-Positive Community-Associated Methicillin-Resistant *Staphylococcus aureus* in Ireland and Internationally using Whole-Genome Sequencing

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Declaration

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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a successful modern pathogen associated with many clinical manifestations including asymptomatic colonisation, superficial skin infections and severe invasive diseases. Over the last three decades, numerous MRSA clones have emerged worldwide within healthcare environments, the community and in livestock settings, many of which encode a wide variety of antimicrobial resistance (AMR) and virulence-associated genes. Panton-Valentine leukocidin (PVL)-negative sequence type (ST)-22-MRSA carrying staphylococcal cassette chromosome *mec* (SCC*mec*) element Type IV is the pandemic healthcare associated (HA)-MRSA clone in several European countries. In Ireland, ST22-MRSA-IV has predominanted in hospitals for over three decades, accounting for approximately 80% of all MRSA bloodstream infections. However, the prevalence of PVL-positive MRSA in Ireland has also gradually increased, with several outbreaks reported in neonatal intensive care units (NICU) and paediatric units. Studies reporting on the population structure of MRSA in Ireland have focused primarily on healthcare associations, neglecting potential reservoirs in the community. The epidemiological work presented in Chapters 3, 4 and 5 investigated the community associated (CA)-MRSA population in Irish hospitals and the association of these lineages with infection outbreaks using high resolution, short-read, Illumina MiSeq-based whole-genome sequencing (WGS). This work underscores the critical importance of on-going surveillance of MRSA to track circulating strains and monitor changing patterns.

The first part of this study investigated multiple distinct outbreaks of PVL-positive CA-MRSA in several Irish hospitals (H1-H3) using WGS. Between 2011 and 2020, a total of 46 suspected outbreak-associated isolates were recovered from 36 individuals across three separate Irish hospitals and submitted to the national MRSA reference laboratory (NMRSARL) for analysis. Thirty-five of these isolates were from patients, one from a healthcare worker (HCW) and the remaining eleven isolates from members of two separate families, at least one member of each had received hospital H2-based treatment for MRSA infection. The 46 isolates underwent whole-genome multi-locus sequence typing (wgMLST) and single nucleotide polymorphism (wgSNP) genotyping. The previously suggested thresholds of ≤24 wgMLST allelic differences and ≤15 SNPs were used for inferring epidemiological relationships and close relatedness between *S. aureus* isolates. The investigation revealed two unrelated PVL-positive ST8-MRSA-IVa outbreaks within two separate Irish hospitals (H1 and H2) over a four-month period between 2017 and 2018. The wgMLST/wgSNP-based minimum spanning trees (MSTs) revealed two distinct clusters, CH1 (8/10 H1 isolates) and CH2 (6/6 H2 isolates). Within each cluster, neighbouring isolates were separated by ≤5 allelic differences; however, ≥73 allelic differences were identified between the two clusters, indicating two separate outbreaks. The occurrence of two distinct, concurrent MRSA outbreaks within a third hospital (H3, maternity hospital) over a 15-month period in 2018–2020 was also confirmed from wgMLST/wgSNP-based MSTs. Two separate isolate clusters, CH3-SCI (14/15 PVL-positive ST5-MRSA-IVc isolates) and CH3-SCII (4/4 PVL-negative ST88-MRSA-V isolates) were identified. Within each cluster, neighbouring isolates were separated by ≤24 allelic differences, whereas the two clusters were separated by 1822 allelic differences, confirming two distinct H3 outbreaks. One of the H3 ST88-MRSA-V outbreak-associated isolates was recovered from a HCW working in this hospital, highlighting the involvement of HCWs in MRSA transmission events. Lastly, intra-familial transmission of PVL-positive ST1-MRSA-V+*fus*+*tirS*+*ccrA1* and PVL-negative ST97-MRSA-V+*fus* was identified in two separate families associated with hospital H2. The presence of three separate clusters (FC1, FC2 and FC2) were observed from the wgMLST/wgSNP-based MSTs, FC1 (4/4 ST1 isolates from family 1 only), FC2-ST1 (4/4 ST1 isolates from family 2) and FC2-ST97 (3/3 ST97 isolates from family 2). Neighbouring isolates within each cluster were closely related and exhibited ≤7 allelic differences. Although intrafamilial transmission was apparent, the detection of ≥48 allelic differences between the clusters indicated no interfamilial transmission between the two separate families.

As part of the CA-MRSA outbreak investigations, surveillance of the CA-MRSA population currently circulating in Irish hospitals was also carried out, primarily focused on CA-MRSA associated with maternity patients, as these are typically healthy individuals with minimal MRSA risk factors, including limited exposure to the healthcare environment. A total of 330 CA-MRSA isolates recovered between 2011 and 2022 from 13 different Irish hospitals (H1–H13) (*N*=326), a Dublin-based General Practitioner (GP) (*N*=2) and a regional GP (*N*=2) were investigated using core-genome multi-locus sequence typing (cgMLST). The population structure analysis revealed a diverse population of CA-MRSA in Irish hospitals, with 32 different STs and 91 different *spa* types identified. The isolates were predominantly ST1 (12.7%), ST5 (22.7%), ST8 (14.5%) and ST22 (18.4%), while the *spa* types mainly included t002 (13.4%), t008 (11%), t127 (12.2%) and t032 (7.3%). This investigation also highlighted a high prevalence of PVL-positive CA-MRSA (91/330, 27.6%) in Irish hospitals, reflective of global trends

where PVL-positive CA-MRSA is continually increasing. The increasing introduction of PVL-positive CA-MRSA into healthcare settings, transmission and association with outbreaks is a serious concern. In recent years, multiple importations of multi-drug resistant (MDR) PVL-positive CA-MRSA clones have been reported, including the ST772-MRSA-V Bengal Bay clone and the ST8-MRSA-IV USA300 clone.

A PVL-positive ST5-MRSA-IVc lineage associated with a protracted 15-month outbreak in H3 was found to exhibit genotypic similarities to previously described 'Sri Lankan clone' isolates which were recently reported as emerging in a large teaching hospital in Sri Lanka, Australia and the UK. The second part of this study investigated the widespread dissemination and diversity of this novel CA-MRSA lineage across numerous disparate geographical regions over a 17-year period using WGS. Thirty PVL-positive ST5-MRSA-IVc isolates (including the 14 H3 outbreak-associated isolates) submitted to the NMRSARL between 2013 and 2022 were investigated. Additional PVL-positive ST5-MRSA-IVc clinical isolates (*N*=56) and WGS datasets (*N*=128 including 46 isolates from the Sri Lankan teaching hospital) recovered from eleven countries including Australia, Czech Republic, Denmark, Germany, Kuwait, Norway, Saudi Arabia, Sri Lanka, Sweden, UAE and the UK between 2005 and 2021 were also investigated. A majority (142/214, 66.4%) of the isolates were from infections, and where detailed metadata were available (168/214; 78.5%), slightly more than half were community associated (85/168, 51%). For comparative purposes, 29 PVL-positive and 23 PVL-negative ST5-MRSA-I/II/IVa/IVc/IVg/V isolates (*N*=41) and WGS datasets (*N*=11) for isolates recovered between 2003 and 2021 from Algeria, Australia, Czech Republic, Denmark, Germany, Ireland, Kuwait, Norway, Saudi Arabia, Senegal, Slovakia, Sri Lanka, Sweden, UAE and the UK were also investigated. The core-genome single nucleotide polymorphism (cgSNP)-based maximum likelihood tree (MLT) grouped 209/214 (97.7%) PVL-positive ST5-MRSA-IVc isolates into Clade I with an average of 110 cgSNPs between isolates. The five remaining PVL-positive ST5-MRSA-IVc isolates grouped into Clade III with an average of 92 cgSNPs. Clade II contained seven PVL-positive ST5-MRSA-IVa comparators, whereas the remaining 45 comparators formed an outlier group. A cgMLST-based MST revealed a comparably low average of 57 allelic differences between the 214 PVL-positive ST5-MRSA-IVc isolates. These isolates were identified as 'Sri Lankan' clone, predominantly *spa* type t002 (186/214, 86.9%) with little population diversity which harboured a similar range of virulence genes and variable antibiotic resistance-encoding genes to one another. The association of Sri Lankan clone isolates with both community and hospital infections in 12 countries spanning 17 years reflects its widespread dissemination internationally and its potential to become a dominant CA-MRSA clone.

The bacteriophage-encoded PVL cytotoxin is considered one of the most significant *S. aureus* virulence factors and is a genetic marker associated with many major CA-MRSA lineages. To date, several PVL-encoding bacteriophages with distinct structural organisations have been described lysogenised into the *S. aureus* chromosome. The third part of this study investigated the PVL-encoding bacteriophage lysogenised in the genome of the PVL-positive ST5-MRSA-IVc Sri Lankan clone. The assembled genomes of the Sri Lankan clone (*N*=214) and comparator (*N*=52) isolates described in the second part of this study were investigated for *pvl*-associated bacteriophage DNA. Twenty-six representative Sri Lankan clone isolates and eight comparator isolates also underwent Oxford Nanopore long-read sequencing and hybrid-assembly with the Illumina-based short-read sequences to further investigate the PVL-encoding phage genome. All 26 Sri Lankan clone isolates and seven Clade II comparators lacked an intact lysogenized PVL-encoding phage genome, but harboured a unique chromosomally integrated 9.6 kb PVL-encoding phage remnant. This remnant exhibited 100% sequence homology with the 3′ junction of the well-characterized PVL-encoding phage φSa2wa.

Using specially designed primers specific to the Sri Lankan clone PVL phage remnant, an *in-silico* PCR analysis was performed to screen the pubMLST database for additional strains harbouring this phage remnant. This pubMLST search resulted in ten PVL-positive ST5-MRSA-IVc, seven PVL-positive ST5-MRSA-IVa and one ST5-MSSA additional assembled genomes harbouring an identical 9.6 kb PVL-encoding phage remnant. A cgSNP-based MLT revealed that the ten ST5-MRSA-IVc pubMLST isolates grouped into Clade I with 209/214 of the ST5-MRSA-IVc Sri Lankan clone isolates with an average of 110 cgSNPs. The seven ST5-MRSA-IVa pubMLST isolates grouped with the seven ST5-MRSA-IVa comparators in Clade II with an average of 78 cgSNPs. The ST5-MSSA isolate branched out next to Clade III Sri Lankan clone isolates exhibiting between 196–240 SNPs to these isolates. These findings suggested that the ST5-MRSA-IVc Sri Lankan clone Clade I/III and the ST5-MRSA-IVa Clade II isolates all arose from a PVL-positive common ancestor harbouring the 9.6 kb phage remnant, very likely a PVL-positive methicillin-susceptible ancestor. The stable chromosomal integration of PVL in the Sri Lankan clone potentially contributes to its widespread dissemination. Additionally, this remnant may be a useful genetic

marker for the Sri Lankan clone, as the earliest Sri Lankan clone study isolate recovered in 2005 also harboured the remnant.

The application of WGS for MRSA epidemiological investigations is highly advantageous as it provides a highly discriminatory tool with unprecedented resolution for accurately tracking spread, monitoring transmission and investigating suspected outbreaks. The increasing prevalence of CA-MRSA, including MDR PVL-positive CA-MRSA lineages in the community and in hospital settings in Ireland is a serious cause for concern. This study highlighted the importance of local and international surveillance in monitoring the emergence and transmission of notable MRSA clones.

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Abbreviations

ACME	Arginine catabolic mobile element
Ak	Amikacin
AMR	Antimicrobial resistance
Ap	Ampicillin
ASC	Ascertainment bias correction
Att	Attachment site
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BSI	Bloodstream infection
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CBA	Columbia Blood Agar
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
cgMLST	Core-genome multi-locus sequence typing
cgSNP	Core-genome single nucleotide polymorphism
CHIP	Chemotaxis inhibitory protein
Cl	Chloramphenicol
CLSI	Clinical Laboratory Standards Institute
cm	Centimetre
Cp	Ciprofloxacin
CRF	Comparator reference isolate
CSO	Central Statistics Office
DDD	Defined Daily Doses
DDUH	Dublin Dental University Hospital
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DR	Direct repeat
e.g.	*Exempli gratia*; for example
EARS-Net	European Antimicrobial Resistance Surveillance Network
EB	Elution buffer
eBURST	Based Upon Related Sequence Types
ECDC	European Centre for Disease Prevention and Control
ED	Emergency department
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EMRSA-15	Epidemic methicillin-resistant *Staphylococcus aureus* 15 variant
ENA	European Nucleotide Archive
Er	Erythromycin

et al.	*Et alia*; and others
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FC	Family cluster
Fd	Fusidic acid
fmol	Fentimolar
G	Gravitational force
GC	Guanine-cytosine
GDP	Gross domestic product
Gn	Gentamicin
GP	General practitioner
GTR	General time reversible
H	Hospital
h	Hour
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HCW	Healthcare worker
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
hla	Alpha-haemolysin
hlb	Beta-haemolysin
HPSC	Health Protection Surveillance Centre
HRP	Horseradish-peroxidase
I	Intermediate
ICU	Intensive care unit
id	Identification
IEC	Immune evasion cluster
Int	Integrase
IPC	Infection Prevention Control
IS	Insertion sequence
ISS	Integration site sequence
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
J	Joining
K/mm^2	Cluster density; thousands of DNA clusters per square milimetre
kb	Kilobase
Kn	Kanamycin
L	Litre
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LFB	Long fragment buffer
Ln	Lincomycin

LV	Latin American variant
Lz	Linezolid
M	Molar
Mb	Mega base
Mbp	Mega base pairs
MDR	Multi-drug resistance
mg	Milligram
$MgCl_2$	Magnesium dichloride
MGE	Mobile genetic element
min	Minute
ml	Millilitre
MLS_B	Macrolide, lincosamide and streptogramin B
MLST	Multi-locus sequence typing
MLT	Maximum likelihood tree
mm	Millimetre
Mp	Mupirocin
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognising Adhesive Matrix Molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MST	Minimum spanning tree
MW2	Mid-Western 2 clone
N	Number
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ND	Not determined
ng	Nanogram
NGS	Next generation sequencing
NICU	Neonatal intensive care unit
NK	Not known
Nm	Neomycin
nM	Nanomolar
NMRSARL	National methicillin-resistant *Staphylococcus aureus* Reference Laboratory
NT	Non-typeable
ONT	Oxford Nanopore Technologies
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PI	Pathogenicity island
pM	Picomolar
PSM	Phenol-soluble modulin

PVL	Panton-Valentine leukocidin
Q	Quality score
Qld	Queensland
R	Resistant
Rf	Rifampicin
RIG	Related isolate group
S	Susceptible
s	Second
S. aureus	*Staphylococcus aureus*
SAK	Staphylokinase
SCBU	Special care baby unit
SCC*mec*	Staphylococcal cassette chromosome *mec* element
SCN	Staphylococcal complement inhibitory protein
SE	Staphylococcal enterotoxin
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
spa	Staphylococcal protein A
SRA	Sequence Read Archive
SSTI	Skin and soft tissue infection
ST	Sequence type
St	Streptomycin
SWP	South Western Pacific
Tb	Tobramycin
TBE	Tris-borate/EDTA buffer
Te	Tetracycline
TGS	Third generation sequencing
TMB	Tetramethylbenzidine
Tn	Transposon
Tp	Trimethoprim
TSST	Toxic shock syndrome toxin
UAE	United Arab Emirates
UK	United Kingdom
USA	United States of America
v	Version
v/v	Volume per volume
VISA	Vancomycin-intermediate *Staphylococcus aureus*
Vn	Vancomycin
VNTR	Variable number tandem repeat
VRSA	Vancomycin-resistant *Staphylococcus aureus*
w/v	Weight per volume

WA	Western Australia
wgMLST	Whole-genome multi-locus sequence typing
WGS	Whole-genome sequence/sequencing
wgSNP	Whole-genome single nucleotide polymorphism
WHO	World Health Organisation
WOAH	World Organisation for Animal Health
ZMW	Zero-mode waveguides
µg	Microgram
µl	Microlitre
-	Negative
%	Percentage
°C	Degrees Celsius
+	Positive
<	Less than
>	Greater than
~	Tilde; approximately
≤	Less than or equal to
≥	Greater than or equal to
α	Alpha
ß	Beta

Publications

Some of the original work presented in this thesis has been published in an international peer-review journal, as listed below. Offprints of the publications are included in the Appendix.

- **McManus B.A., Aloba B.K., Earls M.R., Brennan G.I., O'Connell B., Monecke S., Ehricht R., Shore A.C., and Coleman D.C.** (2021). Multiple distinct outbreaks of Pantone-Valentine leucocidin-positive community-associated meticillin-resistant *Staphylococcus aureus* in Ireland investigated by whole-genome sequencing. *J. Hosp. Infect.* 108:72-80. https://doi.org/10.1016/j.jhin.2020.11.021

- **Aloba B.K., Kinnevey P.M., Monecke S., Brennan G.I., O'Connell B., Blomfeldt A., McManus B.A., Schneider-Brachert W., Tkadlec J., Ehricht R., Senok A., Bartels M.D., and Coleman D.C.** (2023). An emerging Panton-Valentine leukocidin-positive CC5-meticillin-resistant *Staphylococcus aureus*-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by whole-genome sequencing. *J. Hosp. Infect.* 132:8–19. https://doi.org/10.1016/j.jhin.2022.11.015

Chapter 1

General Introduction

1.1 *Staphylococcus aureus*

Staphylococcus is a genus of Gram-positive facultatively anaerobic, coccus-shaped bacteria currently recognised to consist of at least 52 different species and 28 subspecies (Lee *et al.*, 2018). Traditionally, *Staphylococcus* species are categorised into two separate groups based on their ability to produce the coagulase enzyme which clots blood plasma (Foster, 1996). Coagulase binds to and activates prothrombin present in plasma, converting fibrinogen to fibrin and promoting clot formation. Both coagulase-negative staphylococci, such as *Staphylococcus epidermidis* and coagulase-positive staphylococci, like *Staphylococcus aureus* (*S. aureus*) are abundantly present in the microbiome colonising the skin and mucosal membranes of healthy humans and animals (Ahle *et al.*, 2021). *Staphylococcus aureus* is recognised as one of the most important opportunistic bacterial pathogens, which causes significant morbidity and mortality worldwide. The success and widespread dissemination of this pathogen is facilitated by its ability to rapidly acquire/lose a wide range of virulence and antimicrobial resistance determinants during interactions with other commensal staphylococcal species within the microbiome (Otto, 2010). In addition to its use of host fibrinogen to manipulate the human coagulation system, *S. aureus* has evolved overtime to carry a vast arsenal of virulence and immune evasion factors, all of which contribute to its success within the host environment.

1.1.1 *Staphylococcus aureus*: carriage and transmission

Staphylococcus aureus as a commensal bacterium is typically found as part of the normal flora which colonises human and animal mucosal surfaces, particularly the nares, axillae, skin and gastrointestinal tract (Thomer *et al.*, 2016). There are three distinct categories of *S. aureus* carriers in humans including persistent carriers, transient/intermittent carriers and non-carriers. Approximately one in three (20–30%) healthy individuals within the general population are persistently colonised with *S. aureus*, with the anterior nares and skin acting as primary ecological niches (Sakr *et al.*, 2018). A large proportion (60–70%) of the population are categorised as intermittent carriers. In particular, those working within the healthcare environment who are frequently transiently exposed to the bacteria during interactions with colonised/infected patients (Sakr *et al.*, 2018). A small minority (~20%) are non-carriers who almost never carry *S. aureus*. The vast majority of colonised individuals are asymptomatic carriers who go on to transmit the bacteria, most commonly through skin-to-skin contact, but also by contaminating objects in the

environment and by airborne spread (Kong *et al.*, 2016; Sassmannshausen *et al.*, 2016). Persistent carriers who are oftentimes colonised with a higher bacterial load are a high risk group for subsequent infection. Other risk factors for infection include hospitalisation and invasive medical procedures, older age, genetic predisposition, obesity, HIV infection, intravenous drug use and diabetes (Sakr *et al.*, 2018).

As a human pathogen, *S. aureus* frequently invades the skin, soft tissue and bloodstream leading to the onset of clinical manifestations ranging from skin infections, infective endocarditis to bacteraemia and necrotising pneumonia (Pollitt *et al.*, 2018; Tong *et al.*, 2015). The continued emergence and spread of novel *S. aureus* strains has established the organism as a serious threat to public health and the consequences can be observed in countries such as the United States of America (USA) where endemic levels result in significant mortality and morbidity among patients annually (Gajdács, 2019; Kourtis *et al.*, 2019). In 2017 alone, there was an estimated 119,000 *S. aureus* bloodstream infections (BSIs) in the USA, with 20,000 of these resulting in deaths (Kavanagh, 2019; Kourtis *et al.*, 2019).

1.1.2 *Staphylococcus aureus* genome

Comparative analysis of different *S. aureus* strains revealed a common architecture; a typical genome size of ~2.8 mega base pairs (Mbp), a mean GC content of ~32% and ~2,600 protein-coding sequences (J. Wang *et al.*, 2012). The *S. aureus* genome can be divided into two segments: the core-genome and the accessory genome (Bosi *et al.*, 2016; El Garch *et al.*, 2009).

The core genome makes up approximately 75% of the complete genome and contains a set of conserved 'core-stable' genes and 'core-variable' genes shared by the majority of *S. aureus* strains (El Garch *et al.*, 2009). These are species-specific genes and also genes associated with house-keeping functions which are essential for growth and survival.

The accessory genome consists of the remaining 25% and can be defined as genes associated with non-essential functions. The composition of the accessory genome varies significantly between different *S. aureus* strains (Bosi *et al.*, 2016; El Garch *et al.*, 2009). Accessory genomes include mobile genetic elements (MGEs), recently acquired functional genes and non-expressed genes (Rankin *et al.*, 2011). Six distinct types of

MGEs account for approximately 15–20% of the *S. aureus* accessory genome. This includes plasmids, transposons (Tn), pathogenicity islands (PIs), staphylococcal cassette chromosome *mec* elements (SCC*mec*), bacteriophages and insertion sequences (IS). These elements are often acquired through horizontal gene transfer (HGT; transfer of genetic matter between strains) and encode for secondary processes regarding host specificity, immune evasion, toxins, virulence and antimicrobial resistance (Rankin *et al.*, 2011). In recent years the expansion of whole genome sequence projects on the *S. aureus* genome has revealed a considerable variation in the MGE content of different populations of *S. aureus* isolates, highlighting the significance of MGE selection in environmental adaptation (Lindsay *et al.*, 2012).

1.2 Emergence of antimicrobial resistance in *Staphylococcus aureus*

The initial introduction of penicillin as an antimicrobial agent in the early 1940s resulted in significant reductions in staphylococcal infections (Schmidt *et al.*, 2015). Within the context of *S. aureus*, approximately 85% of all strains were susceptible to <0.1 mg/L of penicillin (Schito, 2006). The mechanism of action of this β-lactam antibiotic involves targeting and inhibiting transpeptidase enzyme activity, which typically catalyses the final step in bacterial cell-wall biosynthesis. This prevents cross-linking of the bacterial peptidoglycan chains (Lowy, 2003). By the mid-1940s however, reports of penicillin-resistant staphylococcal strains were well-documented and by the early-1960s, ~80% of all staphylococcal isolates were resistant to penicillin (Lowy, 2003). Widespread resistance was associated with acquisition of plasmid-encoded penicillinase (a β-lactamase enzyme) production by the bacteria resulting in the destruction of the peptide bond of the β-lactam ring which rendered the drug ineffective for clinical use. The spread of penicillin-resistant *S. aureus* was considered a pandemic with resistant strains observed in both the healthcare environment and in the community.

Widespread and inappropriate use of antimicrobial agents is recognised as a major driving force in the rapid evolution and acclimatization of *S. aureus* against various selective pressures (Chambers and Deleo, 2009; Lowy, 2003). Antimicrobial resistance against a wide-range of broad-spectrum antibiotics, including β-lactams, macrolide-lincosamide-streptogramins, tetracyclines, aminoglycosides, fluoroquinolones and glycopeptides is now well-documented in *S. aureus* strains globally (Lowy, 2003). The

emergence of multi-drug resistance (MDR; resistance to at least one agent in ≥3 classes of clinically relevant antimicrobials) has also been reported, posing a major threat to public health.

1.2.1 Methicillin resistance in *Staphylococcus aureus*

Methicillin was designed as a semi-synthetic β-lactam drug to target and circumvent the rise of penicillin-resistant strains of *S. aureus* (Otto, 2012). Much like its predecessor, methicillin also targets and inhibits bacterial cell wall biosynthesis, with the added advantage of resistance to β-lactamases. By the early-1960s, methicillin- and broad-spectrum β-lactam resistance was described in *S. aureus* strains (Fig. 1.1). The acquisition of *mecA* which encodes for an altered penicillin-binding protein 2a (PBP2a) by methicillin-susceptible *S. aureus* (MSSA) led to the rise of methicillin-resistant *S. aureus* (MRSA). The modified PBP2a encoded by *mecA* has a reduced affinity for β-lactam antibiotics (Asghar, 2014). The *mec* gene is usually encoded on the SCC*mec* element which integrates into the bacterial chromosome at the *orfX* locus (Sowash and Uhlemann, 2014). In addition to *mec*, SCC*mec* also encodes for the *mec* gene complex, alongside a site-specific cassette chromosome recombinase (*ccr*) gene complex and a joining (J) region. To date, fourteen types of SCC*mec* elements (I-XIV) have been described with distinct characteristics observed in each (Fig. 1.2). Interestingly, in 2011, a highly divergent homologue of the *mecA* gene, termed *mecC* was described on *SCCmec* element Type XI in Ireland, the United Kingdom (UK) and Denmark (Fig. 1.2) (García-Álvarez *et al.*, 2011; Shore *et al.*, 2011). The *mecC* gene typically exhibits 70% sequence homology to *mecA*, and has been found in MRSA of both human and animal origins (García-Álvarez *et al*., 2011).

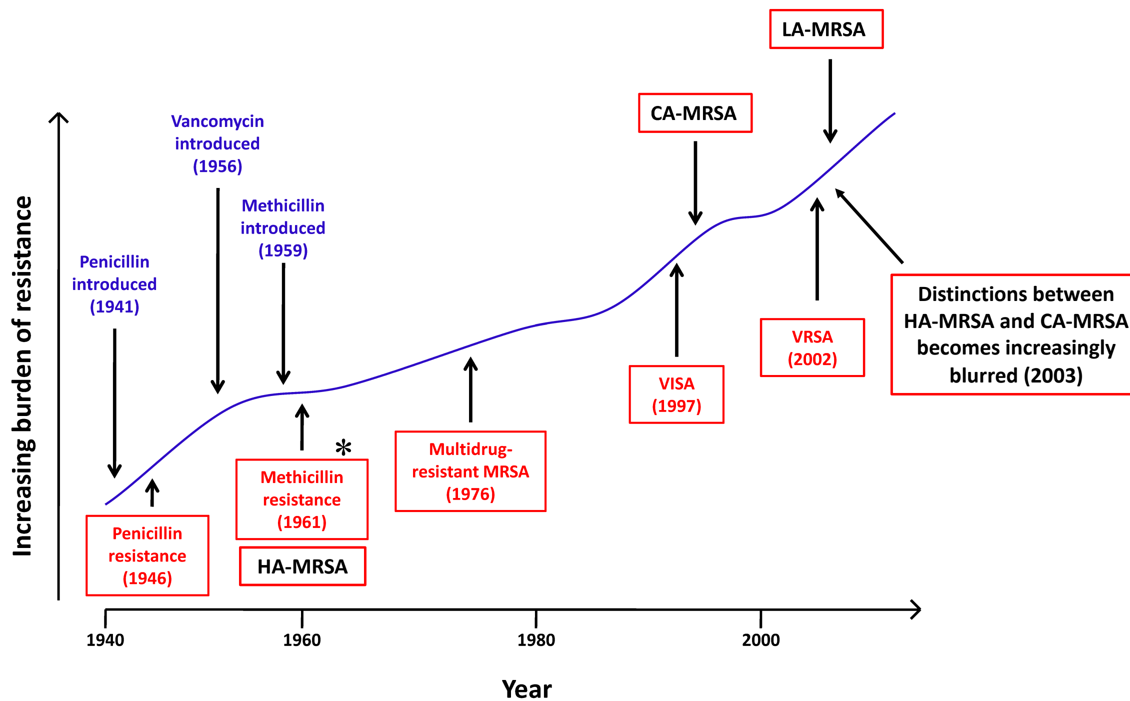


Figure 1.1. Timeline of antimicrobial resistance in *S. aureus*. Abbreviations: MRSA, methicillin-resistant *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; HA-MRSA, healthcare-associated methicillin-resistant *S. aureus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; LA-MRSA, livestock-associated methicillin-resistant *S. aureus*). Adapted from Schmidt *et al*., 2015.

*Recent findings indicate that MRSA emerged 130–200 years ago on hedgehog populations in Denmark and Sweden, and periodically infected humans and cattle. This was long before clinical use of methicillin was introduced in the 1960s (Larsen *et al.,* 2022).

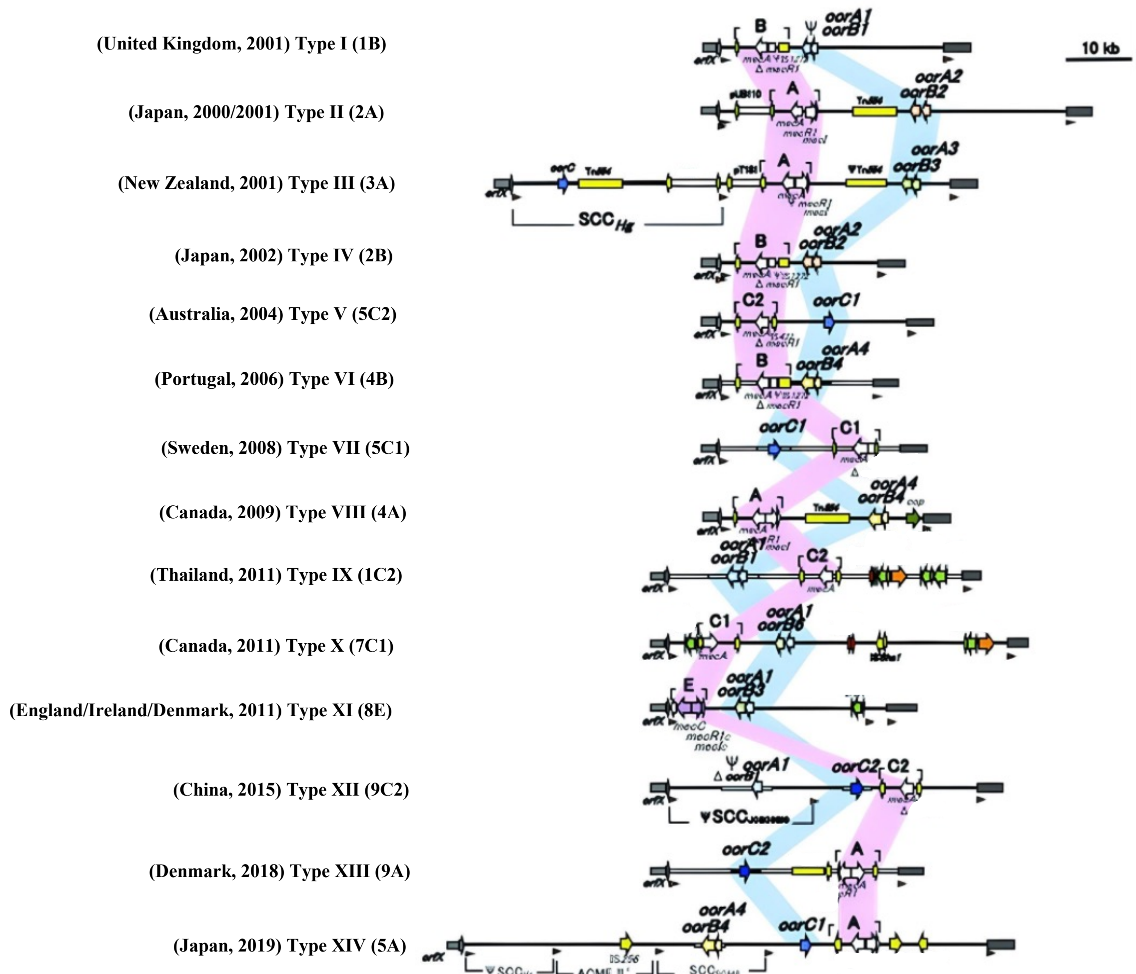


Figure 1.2. Schematic diagram showing the genetic organisation of the 14 SCC*mec* types (I-XIV) currently recognised in *S. aureus.* The pink strip denote the locations of the *mec* complexes. The blue strip denotes the locations of the cassette chromosome recombinase (*ccr*) complexes. Selected genes are indicated as coloured triangles. Country and year of first report are indicated in the brackets. Adapted from Uehara, 2022.

In addition to chromosomal cassette elements such as SCC*mec*, other MGEs including pathogenicity islands, plasmids, bacteriophages and insertion sequences can also encode for virulence factors and antimicrobial resistance determinants to aid MRSA prevalence (Carroll *et al.* 1995; Malachowa and DeLeo, 2010). Currently, the primary recommended 'gold standard' first-line therapy against serious MRSA infections is glycopeptide therapy, particularly vancomycin. In recent years however, vancomycin-resistant and vancomycin-intermediate *S. aureus* (VRSA and VISA) have been isolated in numerous regions worldwide (Otto, 2012).

Methicillin-resistant *Staphylococcus aureus* has emerged as one of the leading pathogens associated with antimicrobial resistance globally. With persistent carriage of *S. aureus* currently at 20–30% within the general population, approximately two in 100 people are colonised with MRSA specifically (American Society for Microbiology, 2015). Additionally, it is a causal agent in the increased incidence of bacterial infections within healthcare settings and in the community (Guo *et al.*, 2020; Morell and Balkin, 2010). Although therapeutic use of methicillin is widely recognised as a driving force behind the rise in nosocomial MRSA, recent studies have shown that emergence of MRSA predates the clinical introduction of methicillin. In 2017, Harkins *et al.*, described the evolutionary history of archetypal MRSA isolates and suggested that acquisition of an ancestral SCC*mec* type I element in the mid-1940s led to β-lactamase-mediated methicillin resistance in *S. aureus* strains long before widespread use of methicillin (Harkins *et al.*, 2017). Another recent study by Larsen *et al.*, in 2022 reported that MRSA possibly emerged 130–200 years ago in hedgehog populations in Denmark and Sweden, and periodically infected humans and cattle too (Larsen *et al.*, 2022). This preceded the introduction of methicillin into clinical practice in the late-1950s.

1.2.2 SCC*mec* element

The highly diverse SCC*mec* element is defined by four main components, which include the presence of the *mec* and *ccr* gene complexes, integration of the element within the SCC integration site sequence (ISS) of the bacterial chromosome and presence of flanking direct repeat (DR) sequences on either side of the integrated SCC*mec* element (Urushibara *et al.*, 2019). At present, the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) recognise grouping of SCC*mec* elements into one of fourteen categories (I-XIV) based primarily on the unique combinations of six *mec* gene complexes, eight *ccr* gene complexes and three J-regions (Fig. 1.2) (Sowash and Uhlemann, 2014; Uehara, 2022; Urushibara *et al.*, 2019). The composition of the *mec* gene complex includes the *mec* gene encoding for PBP2a, regulatory elements which control expression of the *mec* gene (*mecR1-mecI*) and associated insertion sequence (*IS431*). The *ccr* gene complex plays a pivotal role in the inter/intra-species transfer and movement of the SCC*mec* element as it controls integration/excision into the bacterial chromosome. The complex consists of the *ccrAB* or *ccrC* genes and associated open reading frames coding for site-specific recombinases. SCC*mec* elements range in size – from ~20 kb to the longest reported length of 67 kb. Many different subtypes have been defined based on variations in the three J-regions.

Nowadays, *S. aureus* strains carrying SCC*mec* elements constitute significantly to the increasing burden of this highly successful pathogen (Enright *et al.*, 2002). Numerous genetically diverse MRSA lineages have descended independently from the same common ancestral MSSA following acquisition of different variants of SCC*mec* elements and there is clear evidence that in a single event of genetic acquisition, SCC*mec* transforms highly susceptible *S. aureus* strains into MDR pathogens with enhanced virulence and transmissibility (Enright *et al.*, 2002; Rolo *et al.*, 2017). Additionally, the unique combination of SCC*mec* genes within each of the different elements has also been linked with distinct antimicrobial resistance profiles (Mohammadi *et al.*, 2014). Typically, MRSA strains harbouring larger SCC*mec* elements (II and III) exhibit multi-drug resistance, while strains carrying smaller SCC*mec* elements (IV and V) typically express low-level antimicrobial resistance (Udo and Al-Sweih, 2017).

1.2.3 Acquisition of antimicrobial resistance genes

Alongside transfer of MGEs such as SCC*mec*, acquisition of different types of antimicrobial resistance genes is also very common in *S. aureus* strains. Transfer typically occurs through mechanisms of horizontal gene transfer including transformation, bacteriophage transduction or bacterial conjugation (Fig. 1.3) (Álvarez-Martínez *et al.*, 2020); with the latter being the primary mechanism aiding the dissemination of antimicrobial resistance genes in/out of pathogenic *S. aureus* strains (Malachowa and DeLeo, 2010). These resistance genes are typically plasmid-encoded and are transferred from the donor bacterium into the recipient during direct cell-to-cell contact. Numerous distinct antimicrobial resistance genes have been described in *S. aureus* strains (Fig. 1.3), with the most commonly observed conferring resistance against β-lactams (*blaZ*), macrolide-lincosamide-streptogramins (*erm*(A)/(B)/(C)) and tetracyclines (*tet*(K)/(L)/(M)) (Malachowa and DeLeo, 2010).

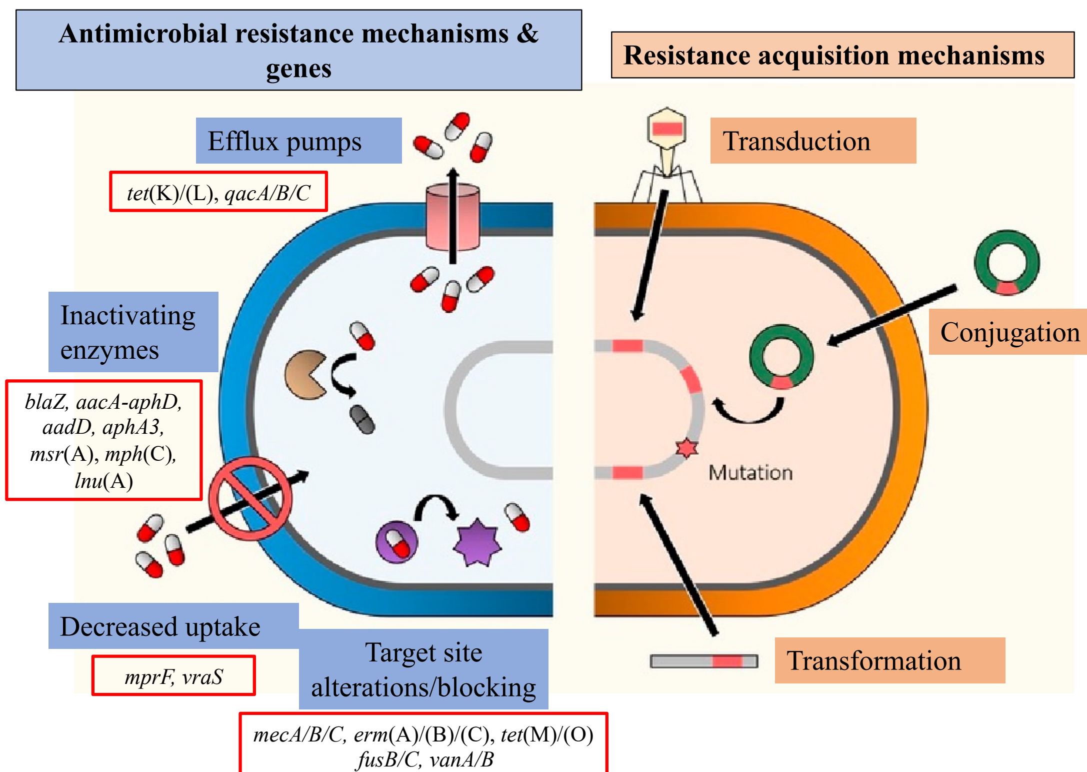


Figure 1.3. Antimicrobial resistance mechanisms and resistance acquisition mechanisms in *S. aureus*. Examples of different types of acquired antimicrobial resistance genes (red) identified in *S. aureus*. Adapted from Álvarez-Martínez *et al.*, 2020.

1.3 *Staphylococcus aureus* virulence factors

In addition to antimicrobial resistance, virulence mechanisms are another important aspect in the pathogenesis of *S. aureus*. The extensive assortment of structural and secreted virulence factors harboured by *S. aureus* strains are typically categorised as toxins, adhesins, degradative enzymes and immunomodulators (Otto, 2012; Watkins *et al.*, 2012). These virulence determinants collectively facilitate entry, attachment and immune evasion of *S. aureus* cells within the host environment during colonisation and infection. Many of these virulence factors are commonly produced by the majority of *S. aureus* strains (e.g., α-haemolysin, phenol-soluble modulins), while others are obtained through MGE exchange between different lineages (e.g. superantigens, Panton-Valentine leukocidin) (Watkins *et al.*, 2012). These MGE-encoded virulence genes are often carried on extra-chromosomal plasmids, transposons or bacteriophages which are either securely retained even as the strain evolves, or are rapidly discarded if they impose a significant fitness cost (Malachowa and DeLeo, 2010). This accounts for the significant diversity often observed between *S. aureus* lineages.

1.3.1 α-haemolysin and phenol-soluble modulins

α-haemolysin and phenol-soluble modulins are two of the most commonly-observed virulence factors carried within the core set of genes of *S. aureus*. These highly cytolytic toxins have previously been described as major virulence determinants found in virtually all *S. aureus* strains. The α-haemolysin/α-toxin is a small β-barrel pore-forming cytotoxin, encoded by the *hla* gene and has a broad range of cellular specificity and is well-characterised for its role in the lysis and destruction of epithelial/endothelial cells and immune cells, including erythrocytes and different types of leukocytes (Berube and Wardenburg, 2013; Vandenesch *et al.*, 2012). Likewise, phenol-soluble modulins (PSMs) are small α-helical peptides which function as cytotoxins efficiently lysing erythrocytes/leukocytes, while also acting as pro-inflammatory agents against host neutrophils (Cheung *et al.*, 2014; Periasamy *et al.*, 2012). Numerous studies have also recently highlighted the role of PSMs in the structuring, stability and detachment of biofilms during infection, particularly on indwelling medical devices.

1.3.2 Superantigens/immune evasion cluster proteins

Superantigens are a notable family of potent immunomodulators associated with increased survival, transmission and pathogenicity of *S. aureus* strains. At least 29 distinct members within this superfamily of virulence toxins have been described within different *S. aureus* lineages, most notably staphylococcal enterotoxins (SEs) and toxic shock syndrome toxins such as TSST-1 (Hu *et al.*, 2021; Hu *et al.*, 2011). Additionally, recent studies suggest that ~80% of all clinically-relevant *S. aureus* strains harbour at least one superantigen gene, the majority of which are MGE-encoded genes carried on plasmids (Holtfreter *et al.*, 2007). With the remarkable ability to recruit and activate a significant number of host T lymphocytes (20–30%) during colonisation and infection, these super-antigenic toxins manipulate normal host immune responses leading to the induction of 'cytokine storms' and apoptosis (Noli Truant *et al.*, 2022).

Another important virulence factor facilitating the survival of *S. aureus* in the host environment is the human-specific immune evasion cluster (IEC). Collectively, these secreted proteins function as immune-modulators counteracting host innate immune responses, particularly through inhibition of neutrophil phagocytosis, chemotaxis and complement activation (Ahmadrajabi *et al.*, 2017; Hau *et al.*, 2015). The cluster consists of staphylococcal enterotoxins (SEA/SEP), chemotaxis inhibitory protein (CHIP), staphylococcal complement inhibitory protein (SCN) and staphylokinase (SAK) (Ahmadrajabi *et al.*, 2017). These virulence factors are typically encoded by genes (*sea/sep, chp, scn and sak*) carried on β-haemolysin-converting bacteriophages, with different *S. aureus* isolates harbouring unique combinations of the genes (Hau *et al.*, 2015). These phages lysogenize the majority of *S. aureus* lineages adapted to human hosts, and integrate into the chromosomal β-haemolysin gene (*hlb*) during lysogenisation resulting in disruption of *hlb*. So far, eight different variants of the IEC genes have been described (Fig. 1.4), with *scn* present within each variant (Chai *et al.*, 2022). The variants are characterised as Type A (*scn, sea, sak, chp*), B (*scn, chp, sak*), C (*scn, chp*), D (*scn, sak, sea*), E (*scn, sak*), F (*scn, chp, sak, sep*), G (*scn, sak, sep*), and H (*scn* only) (Fig. 1.4) (Wamel *et al*., 2006). In the current study, three novel IEC variants were also detected. This included Novel type 1 (*sep* only), Novel type 2 (*scn, sea*, *sak, sep*) and Novel type 3 (*sak, sep*).

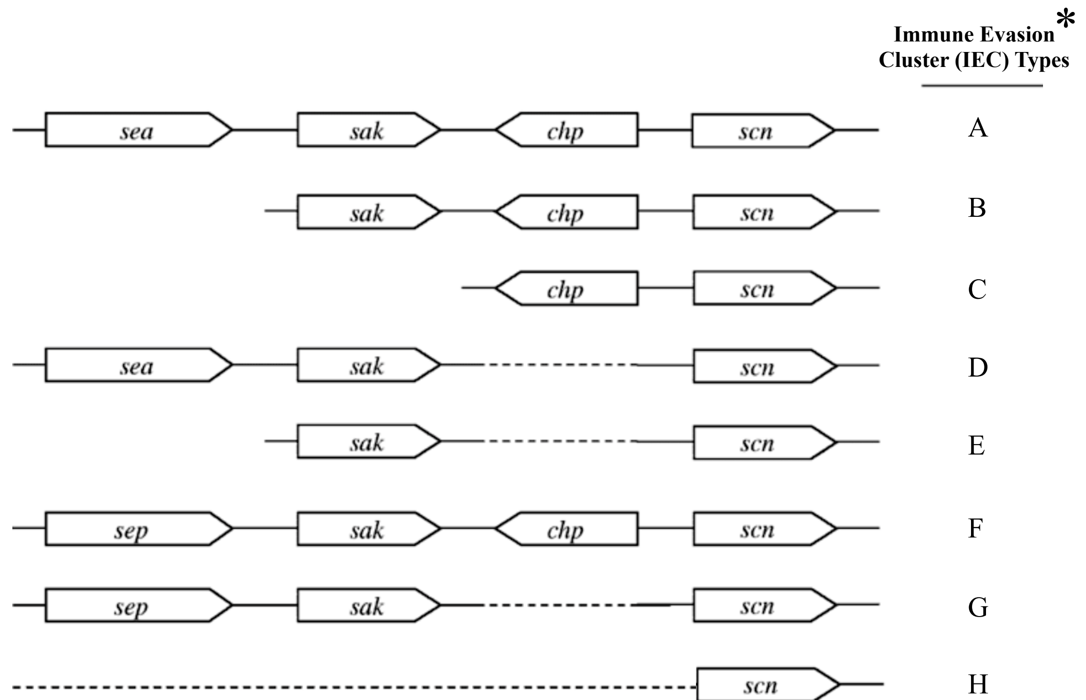


Figure 1.4. Schematic diagram of the eight different immune evasion cluster (IEC) types (A–H) recognised in *S. aureus*. Arrow boxes indicate the presence of *sea, sak, chp*, and *scn* genes. Dashed lines indicate the absence of one or more of these genes. Adapted from van Wamel *et al*., 2006.

*Three novel IEC variants were detected in the current study. This included Novel type 1, *sep* only; Novel type 2, *sak, scn, sea and sep*; Novel type 3, *sak and sep.*

1.3.3 Panton-Valentine leukocidin

Panton-Valentine leukocidin (PVL) is a bicomponent exotoxin expressed by at least 2–5% of all *S. aureus* isolates, including MRSA (Lina *et al.*, 1999). With structural similarities to α-haemolysin toxins, PVL also forms cytolytic pores in the leukocyte cell membrane after binding to complement receptors leading to cell destruction and tissue necrosis within the host (Kaneko and Kamio, 2004; Lina *et al.*, 1999). The PVL toxin is encoded by two genes (*lukF-PV, lukS-PV*) which are located in the genomes of a range of lysogenic bacteriophages integrated into the *S. aureus* chromosome (Fig. 1.5) (Lina *et al.*, 1999). Eight distinct types of these PVL bacteriophages bearing either an icosahedral morphology or elongated-head shape have been described (ΦPVL, Φ108PVL, Φ7247PVL, ΦSa2958, ΦSa2USA, ΦSa2MW, ΦSLT, ΦTCH60), and transmission between staphylococcal species is suggested to be limited by phage/bacterial specificity factors (Boakes *et al.*, 2011). Although the overall importance of the toxin as a virulence determinant in pathogenicity is often debated, many studies have associated PVL-producing *S. aureus* isolates with more serious, invasive infections and also recurrent skin/soft tissue infections.

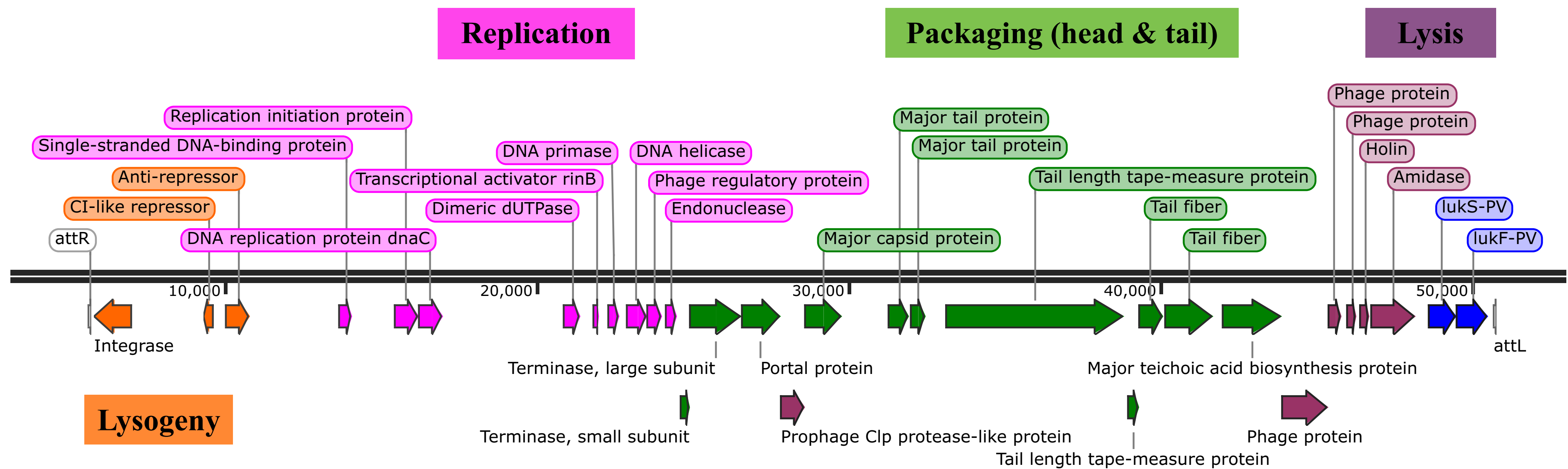


Figure 1.5. Schematic diagram of a typical Panton-Valentine leukocidin (PVL)-encoding bacteriophage lysogenised in the *S. aureus* chromosome. The phage is approximately 40,000–50,000 bp in size. The *pvl* genes encode for the PVL cytotoxin, a virulence factor commonly associated with CA-MRSA strains. The typical structure of the bacteriophage includes the lysogeny module (highlighted in orange), DNA replication and transcription genes (highlighted in pink), the head and tail packaging region (highlighted in green), lysis module (highlighted in dark purple) and the *lukS/F*-PV genes region (highlighted in blue). Attachment sites (*attL* and *attR*) are typically present at the proximal and distal junction ends of the phage/chromosomal regions. Created using SnapGene v6.0.6 (https://www.snapgene.com).

1.4 Traditional molecular typing techniques

Molecular typing facilitates epidemiological surveillance of both methicillin-susceptible and methicillin-resistant *S. aureus*. Focusing primarily on discreet genotypic variabilities between isolates, molecular typing can be used to define transmission pathways, inform Infection Prevention and Control (IPC) measures, monitor prevalence, track spread and to investigate the emergence of novel antimicrobial-resistant strains (Al-Obaidi *et al.,* 2018; Trindade *et al.,* 2003). Traditionally, discrimination of *S. aureus* was achieved through the use of typing tools that differentiate isolates based on phenotypic traits such as antimicrobial susceptibility patterns or phage typing patterns (Collins *et al.,* 1984). These methods had limited discriminatory powers and were replaced by molecular typing approaches such as DNA fingerprinting (e.g., pulsed-field gel electrophoresis; PFGE), variations within the coding sequences of segments of seven core housekeeping genes (multi-locus sequence typing; MLST), variations in the repeat units within the staphylococcal protein A gene (*spa* typing) or characterisation of the SCC*mec* element (SCC*mec* typing). High-throughput comparison of antimicrobial and virulence genetic determinants by DNA microarray profiling has also been widely employed (Al-Obaidi *et al.,* 2018; Asadollahi *et al.,* 2018). Undeniably, these standardised typing systems with significant discriminatory power and reproducibility have been invaluable for epidemiological investigations on *S. aureus* and have facilitated the collective analysis of strains worldwide *via* internet-based databases (Trindade *et al.,* 2003).

1.4.1 Pulsed-field gel electrophoresis

Developed in the early 1980s, PFGE used to be referred to as the 'gold standard' DNA-based molecular typing technique for genotyping *S. aureus* isolates, due to its high resolution and sensitivity (Le Bourgeois *et al.,* 2015; Sharma-Kuinkel *et al.,* 2016). During this period, the PFGE approach provided higher typeability and greater discriminatory power than other commonly employed typing tools, including phage typing and ribotyping (Gibson *et al.,* 1995; Tenover *et al.,* 1994). The PFGE process involves lysing the bacterial cell in agarose plugs to isolate its intact bacterial genomic DNA molecule (Tenover *et al.,* 1994). This is followed by digestion into high molecular weight fragments (~10 Mb) using restriction endonucleases that cleave DNA infrequently (e.g., *Sma*I). Fragments are resolved/separated according to size in agarose gels by reorienting the electrical field between spatially-distinct electrodes over an extended period of time. The migration of the DNA fragments through the gel at varying

speeds results in a unique DNA banding pattern, commonly referred to as 'DNA fingerprints'. The gel can then be visualised using computer software programs, such as GelCompar and BioImage which analyse and identify polymorphisms in the DNA band patterns. Variations in the fingerprints of different isolates are compared by the programs to determine close relatedness and confirm clonality (Sharma-Kuinkel *et al.,* 2016). Despite its high discriminatory power and widespread availability, the process lacked adequate inter-laboratory reproducibility, as well as being labour-intensive (2–3 days). Currently, although standardisation of guidelines and data interpretation techniques have greatly reduced these limitations, use of PFGE for molecular typing of *S. aureus* has been largely replaced by state-of-the-art whole-genome sequencing (WGS)-based approaches.

1.4.2 Multi-locus sequence typing

Multi-locus sequence typing is a molecular typing approach based on Sanger sequencing technology. It allows for the systematic discrimination of *S. aureus* isolates based on the polymerase chain reaction (PCR) amplification and subsequent sequencing of 450–500 bp internal fragments of seven well-conserved house-keeping gene loci (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*) (Enright *et al.,* 2000). The process unambiguously characterises *S. aureus* isolates based on nucleotide variations within each of these seven genes, and assigns a specific integer for each distinct genotype (Maiden *et al.,* 2013). The integers for each of the seven loci are combined to generate an allelic profile and each distinct allelic profile is assigned a uniquely corresponding integer referred to as a ST. Isolates with the same ST are genetically indistinguishable at each of the seven loci included in the MLST scheme (Maiden *et al.,* 2013; Robinson and Enright, 2004). Sequence types which differ at only one of the seven gene loci are typically defined as the same CC (Enright *et al.,* 2000). Over the years, a MLST database (https://pubmlst.org/ saureus/) consisting of allelic profiles of 8150 distinct STs (database accession update: 08-03-2023) has been collectively populated by researchers worldwide (Robinson and Enright, 2004). As the traditional MLST technique uses only seven stable core-genome housekeeping genes, the resolution of this tool is quite limited. For suspected outbreak investigations of closely-related strains, use of MLST has been replaced by more-discriminatory genotyping techniques, such as *spa* typing.

1.4.3 *spa* typing

The *spa* typing method is a single-locus typing technique based on sequencing of a highly-conserved gene (*spa*). The *spa* gene encodes for staphylococcal protein A, a key cell surface immune evasion virulence factor secreted by the majority of *S. aureus* isolates (Hallin *et al.,* 2007; Sowash and Uhlemann, 2014). The 3' end of the *spa* gene carries a polymorphic X region which contains variations in the number of tandem repeats and base sequence within each repeat. This is known as the variable number tandem repeats (VNTR), and each sequence motif is typically 24 bp in size (Hallin *et al.,* 2007). The *spa* typing tool, much like MLST also employs PCR-amplification and Sanger sequencing for this highly discriminatory molecular typing process. Specialised software assigns each individual repeat variant a unique repeat code and isolates are assigned a '*spa* type' based on the order/succession of specific repeats. Following assignment of a novel *spa* type to an *S. aureus* strain, the information is documented and uploaded into a publicly accessible web-based depository (http://spaserver.ridom.de) which currently contains 20,954 *spa* types and 835 *spa* repeats from 159 different countries (database accession update: 08-03-2023). *Spa* typing is commonly utilised in clinical and epidemiological investigations as a rapid, cost-effective alternative to MLST (Sowash and Uhlemann, 2014). It is highly concordant with MLST as *spa* types commonly correlate with CCs and STs defined by MLST (O'Hara *et al.,* 2016). This technique has enabled investigations of local *S. aureus* hospital outbreaks and global surveillance studies where high discriminatory power is needed to differentiate between genetically similar strains (Hallin *et al.,* 2007).

1.4.4 SCC*mec* typing

SCC*mec* typing is a molecular typing tool which takes advantage of the diversity in genomic content, structural composition and organisation of SCC*mec* elements, particularly in the *mec* and *ccr* gene complex (Asghar, 2014; Hallin *et al.,* 2007; Shore *et al.,* 2012). The presence of allotypic differences in the *mec* complex (Class A, B, C1, C2 and D) and in the *ccr* complex (*ccrAB1, ccrAB2, ccrAB3, ccrAB4* and *ccrC*) allows for classification of SCC*mec* elements into types (Fig. 1.2) (IWG-SCC, 2009). Additionally, polymorphisms in the J-regions (J1-J3), the non-essential SCC*mec* component located between *mec* and *ccr* gene complexes are also employed for defining SCC*mec* subtypes (IWG-SCC, 2009; Sowash and Uhlemann, 2014). Oliveira and de Lencastre, 2002, designed a single-step multiplex-PCR (M-PCR) approach to assign

SCC*mec* subtypes based on the structural variations in eight gene loci (A-H) within the J-region. A more complete typing method based on a set of six M-PCR reactions was developed by Kondo *et al*., 2007, whereby allotypic differences in *mec*, *ccr* and also in the J-regions are identified and SCC*mec* types are assigned. To date, fourteen distinct SCC*mec* types (I-XIV) and numerous subtypes have been described in MRSA clones (Fig. 1.2), but the M-PCR strategy for SCC*mec* typing still only covers a subset of these (Saber *et al.,* 2017; Sowash and Uhlemann, 2014; Urushibara *et al.,* 2019). To combat this limitation in SCC*mec* typing and subtyping, an *in silico* approach based on WGS data is now widely available (https://cge.cbs.dtu.dk/services/SCCmecFinder/) (Kaya *et al.,* 2018).

1.4.5 DNA microarray profiling

DNA microarray profiling is a highly effective tool for *S. aureus* genotyping due to its discriminatory power for outbreak investigations (Monecke *et al.,* 2008). It is often proposed as a suitable alternative to both MLST and SCC*mec* typing as it examines a large number of genes and markers in the genome as opposed to only a select number of genes. The abundant availability of whole genome sequences for a wide-range of *S. aureus* strains in recent years has led to the advancement of many genotyping techniques, including microarray profiling (Monecke *et al.,* 2008). Notably, microarray techniques have been expanded with oligonucleotide probes to allow for identification of additional virulence and pathogenicity-associated genes. The *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany) is one of the most extensively used microarray tools for strain identification. This array permits the detection of 333 target sequences which characterise approximately 170 clinically-relevant resistance and virulence genes (Shittu *et al.,* 2015; Shore *et al.,* 2012). Based on these markers, DNA microarray profiling identifies isolate STs, antimicrobial resistance and virulence genes, and also SCC*mec*-associated genes (Shittu *et al.,* 2015).

1.5 Whole-genome sequencing

Although traditional molecular typing techniques have been shown to be well-suited highly discriminatory and reliable tools for monitoring *S. aureus* transmission and circulation, these techniques are still quite limited in their abilities and focus primarily on only a single specific polymorphic gene or target element. Rapid advancements in

next-generation sequencing (NGS) technologies in recent years have resulted in the replacement of conventional molecular typing tools with novel whole-genome based systems in many diagnostic and research facilities worldwide. These whole-genome approaches have completely revolutionised how *S. aureus* epidemiological investigation and surveillance is performed, offering significantly higher resolution, increased inter-laboratory reproducibility and accuracy for strain characterisation (Durand *et al.,* 2018). With its unmatched discriminatory power, WGS can distinguish between isolates on the basis of STs, *spa* types, SCC*mec* types, serotypic/genotypic differences, virulence factors, antimicrobial resistance and can be used to decipher new and emerging MGEs (Deurenberg *et al.,* 2017; SenGupta *et al.,* 2014). In contrast to Sanger-based sequence genotyping, NGS generates more data, offers a single protocol approach for strain identification and also makes genotyping at the single nucleotide level feasible (Deurenberg *et al.,* 2017; Quainoo *et al.,* 2017). The evolution and era of sequencing technologies can be divided into three separate categories commonly referred to as generations; First Generation, Next/Second Generation and Third Generation Sequencing (Park and Kim, 2016).

1.5.1 First generation sequencing

First generation sequencing approaches typically represents the two DNA sequencing techniques developed in the 1970s; Maxam-Gilbert sequencing that involves a chemical degradation method whereby the target DNA is radio-labelled and split into four chemical cleavage reactions (sequencing-by-cleavage) and Sanger sequencing (chain-termination method), which is based on a sequence-by-synthesis approach (Heather and Chain, 2016; Park and Kim, 2016). In the latter, fluorescently-labelled chain-terminating nucleotide sequences selectively incorporated by a DNA polymerase during the *in-vitro* replication of a DNA template are detected (Heather and Chain, 2016). Both of these methods provided the necessary foundations which led to the development of all the other generations of sequencing technologies utilised today. Sanger sequencing remains the only first generation technique still routinely employed for individual gene sequencing (Dewey *et al.,* 2012). Although it is labour-intensive and only sequences a few hundred base pairs at a time (<1000 bp), it generates highly accurate and easily interpretable data (Heather and Chain, 2016).

1.5.2 Second/Next (short-read) generation sequencing

The rise in WGS for comprehensive genomic analyses over the last decade has led to the replacement of many traditional DNA sequencing approaches with second/next generation sequencing technologies (Quail *et al.,* 2012). These technologies produce millions of short sequence reads with a size of 50-500 bp in a relatively shorter timeframe in comparison to older methods such as Sanger sequencing (Park and Kim, 2016; Quail *et al.,* 2012). To achieve this, the entire genome is broken down into smaller fragments which are then amplified and subsequently sequenced (Heather and Chain, 2016). Such NGS platforms include Roche 454 (Branford, Connecticut, USA), Applied Biosystems/Life Technologies' SOLiD (Grand Island, New York, USA), Ion Torrent's PGM (Guilford, Connecticut, USA) and Illumina systems (Eindhoven, The Netherlands) which all serve as high-throughput sequencing technologies for generating large volumes of data rapidly and provide high-resolution insights into the complete genome of organisms (Heather and Chain, 2016; Park and Kim, 2016). The Illumina platform in particular has undergone significant advancements over the years and currently dominates the high-throughput sequencer market (Quail *et al.,* 2012). Within many clinical microbiology laboratories nowadays, researchers can study complete genomic profiles of numerous isolates in a matter of days (SenGupta *et al.,* 2014). These advancements in Illumina's technology and its corresponding bioinformatics-based analysis tools have greatly improved the output and analysis speed of this technology making it an ideal approach for genotyping bacterial strains (Quainoo *et al.,* 2017).

The Illumina sequencing process typically involves four basic steps (Fig. 1.6), starting with library preparation whereby DNA sequencing libraries are prepared by random fragmentation of high molecular weight DNA into shorter fragments of specific sizes (Heather and Chain, 2016; Qin, 2019). Adapter sequences are then annealed/ligated onto the ends of these fragments (Qin, 2019). The use of preparation kits provided by Illumina allows for the fragmentation and ligation reactions to be combined into a single step commonly referred to as tagmentation (Feng *et al.,* 2018). The library sample is then denatured, loaded into a reagent cartridge and inserted into the Illumina sequencer. Within the sequencer, the cluster generation process begins whereby the adapter-ligated single-stranded DNA fragments bind onto the surface of a flow cell with oligonucleotide primers which are complementary to the library adapters (Fig. 1.6) (Besser *et al.,* 2018; Qin, 2019). The free ends of the fragments bind to the flow cell through base pairing and

each bound fragment undergoes repeated bridge PCR amplification that generates distinct clusters (Heather and Chain, 2016). Following cluster generation, the template fragments can then be sequenced (Fig. 1.6). To do this, fluorescently-labelled nucleotides which correspond to each of the four nucleotide bases are incorporated into new DNA strands which are complementary to the initial template (Besser *et al.,* 2018; Heather and Chain, 2016). During each sequencing cycle, a fluorescent labelled nucleotide is added to the growing DNA strand of each fragment cluster, a laser excites this newly incorporated nucleotide and the fluorescence signals emitted are detected by an optic scanner (Dewey *et al.,* 2012). These nucleotides also act as terminators of synthesis for each reaction, which are removed after detection occurs allowing for the next sequencing cycle to commence (Besser *et al.,* 2018; Suaya *et al.,* 2014). Illumina currently offer a wide range of sequencing platforms which include the MiSeq, HiSeq, NovaSeq, NextSeq and MiniSeq, all with varying levels of throughput (Besser *et al.,* 2018). The MiSeq in particular is one of the most frequently employed systems as it generates high-quality short reads at an affordable run cost while providing the greatest time benefit (Besser *et al.,* 2018).

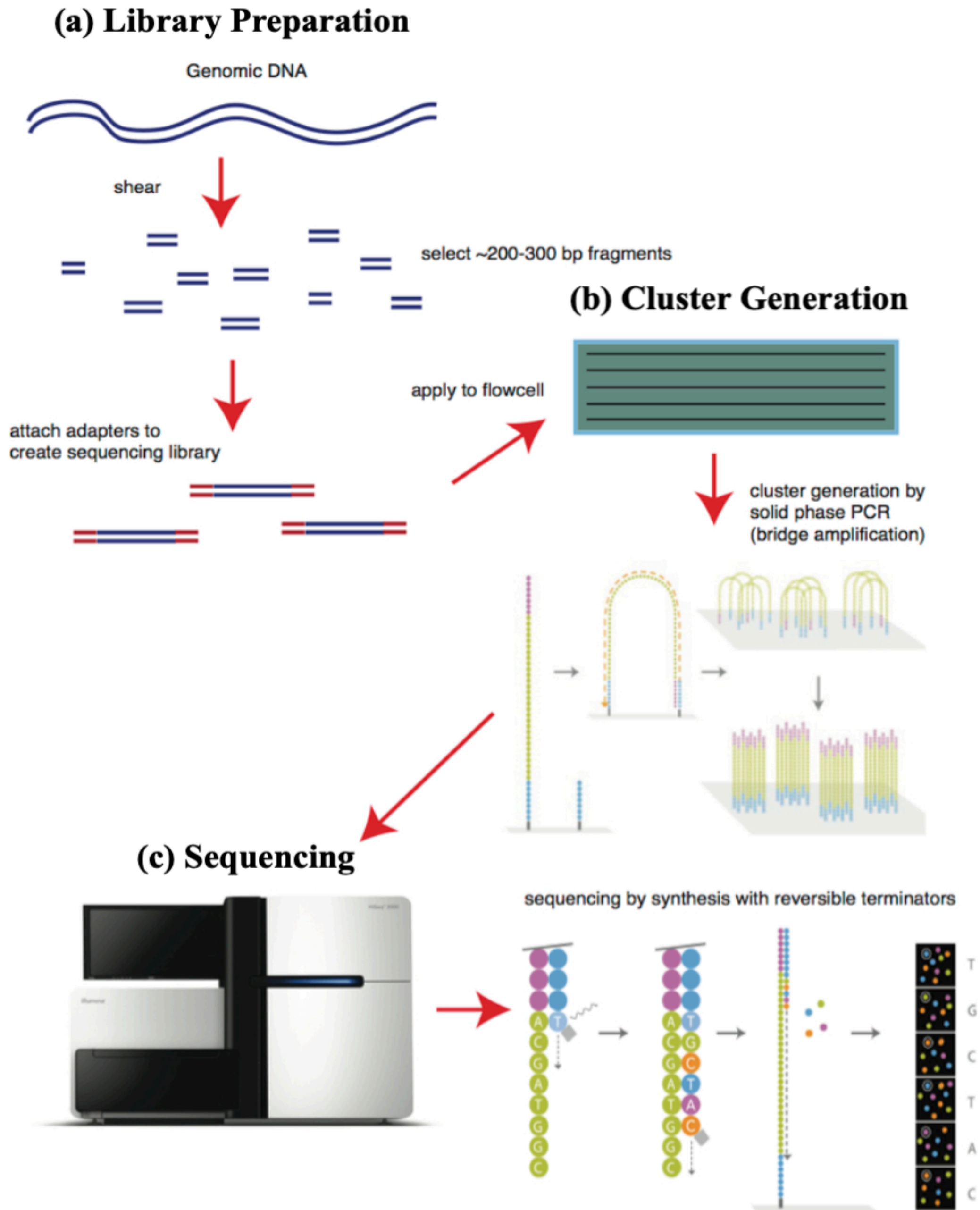


Figure 1.6. Schematic diagram of the Illumina sequencing technology. The process follows four basic steps: genomic DNA library preparation, cluster generation through PCR bridge amplification, sequencing-by-synthesis and data analysis. Adapted from Brown, 2012.

1.5.3 Third generation (long-read) sequencing

Shortly after the introduction of NGS, third generation sequencing (TGS) systems also emerged on the market. In contrast to the base-by-base sequencing provided by NGS platforms, TGS technologies on the other-hand are often referred to as single-molecule long-read sequencing whereby the genome is directly sequenced in real-time as a single long DNA molecule and not broken down or amplified (Dewey *et al.,* 2012; Park and Kim, 2016). Currently, the most popular commercially available third generation platforms include Pacific Biosciences' (PacBio) Single Molecule Real Time (SMRT) sequencing (Menlo Park, California, USA) and Oxford Nanopore Technologies' (ONT) ION systems (Littlemore, Oxford, UK) (Heather and Chain, 2016).

The PacBio SMRT system, which was released for commercial use in 2011 closes and circularises a double-stranded DNA fragment by ligating hairpin adapters onto the strand ends to create a single-strand template referred to as a SMRTbell (Ardui *et al.,* 2018; Rhoads and Au, 2015). Library preparation also involves annealing the adapter with a primer and a polymerase before loading onto a SMRT cell. This specialised flow-cell consists of 150, 000 microscopic observation chambers/wells, known as the zero-mode waveguides (ZMW) which allows for real-time detection of the single-molecule DNA undergoing sequencing. The sequencing-by-synthesis reaction occurs at the bottom of each ZMW chamber and involves binding the adapters with the immobilised polymerase to replicate the target DNA molecule. Replication involves incorporation of four fluorescently-labelled nucleotides into the newly synthesised DNA, resulting in production of distinct fluorescence signals upon excitation. As each nucleotide passes through the polymerase, the signal produced is detected by a laser and the colour/duration of each light pulse emitted is recorded by a camera. The pulses corresponding to each ZMW are then interpreted as a sequence of bases or a continuous long read sequence.

Oxford Nanopore took high-throughput long-read sequencing to the next level with the introduction of the MinION sequencer in 2014 (Ambardar *et al.,* 2016; Lu *et al.,* 2016). This inexpensive, pocket-sized universal serial bus (USB)-device operates by measuring small base-specific changes in the electrical conductivity generated as the DNA template strand passes through a biological nanopore. After library preparation where DNA fragments also undergo adapter-ligation and end-repair, the sample is loaded onto a flow-cell. This process also takes place within a flow-cell containing two ionic electrolyte-

filled chambers separated by a thin high-voltage membrane. When a voltage bias is applied across the membrane, an ionic current is produced which flows through the nanopore to translocate a DNA molecule. As the DNA moves through the nanopore, the ionic current is disrupted and the change in current is measured as a characteristic 'squiggle plot' by a sensor. The squiggle can then be processed and interpreted by the MinKNOW software. The MinION flow-cell contains 512 channels with 4 nanopores each, allowing for sequencing of up to 512 independent DNA molecules simultaneously. A significant drawback for both PacBio SMRT and ONT MinION long-read sequencing is that although the systems provide access to longer single-molecule DNA fragments, the sequencing error rates are significantly high (10–20%) and affect the accuracy of the raw reads (Ambardar *et al.,* 2016). To combat this limitation, an innovative process known as hybrid assembly was recently developed. This bioinformatics-based approach involves scaffolding fragmented NGS short reads with longer TGS data to increase overall accuracy and allow for error corrections (Heather and Chain, 2016; Park and Kim, 2016).

1.6 NGS-based *S. aureus* typing and epidemiological investigations

As it stands, short-read NGS is still the primary choice for WGS-based genotyping and characterisation of closely-related strains due to the higher sequencing error rates of TGS platforms (Park and Kim, 2016). Although these systems are still not as affordable as conventional molecular typing techniques, NGS-based genotyping is highly advantageous in its ability to provide a wide-range of molecular, clinically-relevant information in a relatively short timeframe (Deurenberg *et al.,* 2017). The Illumina NGS MiSeq benchtop sequencing platforms is currently one of the most popular sequencing systems in research and clinical settings due to its relatively lower cost, low error rates and high throughput. Following completion of an MiSeq Illumina sequencing run, forward and reverse FASTQ files (for paired-end reads) are generated for each sample included in the library (Holmes *et al.,* 2018). The FASTQ files containing all sequence reads and quality data associated with each sample are usually stored in Illumina's BaseSpace cloud platform (Deurenberg *et al.,* 2017). Current commercially available and user-friendly tools for analysing these files and genotyping (e.g., core-genome and whole-genome MLST) include Seqsphere (Ridom GmbH, Münster, Germany) and BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Sequence assembly,

quality assessment, data analyses and visualisation can all be performed within these software programs (Deurenberg *et al.,* 2017; Holmes *et al.,* 2018).

Core-genome and whole-genome multi-locus sequence typing (cgMLST and wgMLST, respectively) employ comprehensive MLST schemes to analyse nucleotide sequence data from the thousands of genes within the core and/or accessory genomes. These WGS-based genotyping tools have higher resolution capabilities and greater discriminatory power than conventional MLST (Bosi *et al.,* 2016; Rankin *et al.,* 2011). Although the advantages of WGS-based MLST over conventional MLST is quite evident, it is still important for newly sequenced isolates to be analysed within the context of older isolate collections present in the pubMLST database (Inouye *et al.,* 2012). This database stores a significant amount of information which serve as a framework for describing newly sequenced isolates. An advantage of the novel cg/wgMLST system is that there is backwards compatibility between them and older MLST schemes, thus novel strains and lineages can also be defined using previously assigned ST and CC identifiers determined by conventional MLST (Deurenberg *et al.,* 2017; Inouye *et al.,* 2012).

1.6.1 cgMLST

For epidemiological investigations of closely-related *S. aureus* isolates based on WGS, conventional MLST has been expanded and modified further from its initial usage of seven house-keeping gene loci to include a wider range of 1,861 pre-defined genes from the stable core-genome (Earls *et al.,* 2018; Leopold *et al.,* 2014). Core-genome MLST is a standardised, highly reproducible process developed by Leopold *et al.,* in 2014 and involves pairwise gene-by-gene comparisons of each locus to establish an allelic profile for each isolate (Leopold *et al.,* 2014). Although its resolution far surpasses that of traditional MLST, it is still not sensitive enough for distinguishing outbreak-associated isolates (Quainoo *et al.,* 2017). As a result, its use in outbreak investigations is limited, however it is a valuable tool when comparing groups of isolates acquired over an extended period from different geographical regions where accessory genomes are of little relevance (Earls *et al.,* 2018). There are currently no conclusive thresholds or benchmarks for assigning isolate relatedness based on cgMLST allelic differences, but previous studies have suggested that an allelic difference threshold of ≤24 is a suitable parameter for defining close relatedness between isolates (Earls *et al.,* 2018; Schürch *et al.,* 2018).

1.6.2 wgMLST

Further expansion of the MLST scheme led to the development of wgMLST, which not only includes core-genome loci as in cgMLST, but also incorporates accessory genomes to analyse a total of 3,904 loci in a standardised *S. aureus* wgMLST scheme (Earls *et al.,* 2018). The inclusion of accessory gene loci in the wgMLST scheme ensures any relevant information is not overlooked when investigating relatedness of genetically similar isolates. The wgMLST application is advantageous for local epidemiological investigations as it provides a complete breakdown of the genome capturing allelic variants and sequence features (Earls *et al.,* 2018; Kingry *et al.,* 2016). Due to its higher typing resolution, wgMLST is often employed for gene-by-gene comparisons during MRSA outbreak analysis and strain tracking. The scheme also takes an allelic difference of ≤24 as an appropriate threshold for defining isolate relatedness (Kingry *et al.,* 2016; Schürch *et al.,* 2018).

1.6.3 Analysis of single nucleotide polymorphisms

The development of highly advanced WGS typing techniques also allows for distinction between isolates based on single nucleotide variations/polymorphisms (SNVs/SNPs) (Al-Obaidi *et al.,* 2018). The variations occur at a single base position in the DNA sequence as a result of point mutations which gradually accumulate over time. Mutation rates vary between different species, and current estimations suggest that the rate of mutation in MRSA is between 2–10 SNPs in a single genome per year. Consequently, this high resolution genotyping tool is often employed for tracking transmission and confirming close-relatedness between isolates (Al-Obaidi *et al.,* 2018). The analysis uses a suitable reference genome that is closely related to the query isolates to detect SNP distributions amongst the isolate collection under investigation. This provides higher-level discrimination compared to most other typing techniques (Schürch *et al.,* 2018). Similar to the wgMLST scheme, SNP analysis is also performed based on the whole genome, however in this instance, the suggested threshold applied for defining isolate relatedness is ≤15 wgSNPs (Schürch *et al.,* 2018).

1.7 Epidemiology of MRSA

With the initial discovery of methicillin resistance in the early 1960s, MRSA colonisation and infection were predominantly attributed to hospitals and other healthcare environments only. By the late 1990s however, MRSA strains causing infections in

young, healthy individuals with no prior exposure to hospitals within the general population were reported steadily (Dukic *et al.*, 2013). Clear distinctions were noted in the pathogenicity, antimicrobial resistance patterns and virulence properties of these MRSA strains causing infections within healthcare settings versus those causing community infections (Dukic *et al.*, 2013; Lakhundi and Zhang, 2018). Accordingly, strains were categorised as either healthcare-associated (HA-MRSA), community-associated (CA-MRSA), and more recently livestock-associated (LA-MRSA) based primarily on the types of SCC*mec* elements and range of antimicrobial resistance genes harboured, and also in carriage of PVL-encoding genes between CA- and HA-MRSA strains (Fig. 1.1) (Lakhundi and Zhang, 2018). Likewise, distinct epidemiological and genotypic properties were recognised in LA-MRSA strains. The distinctions proved effective for surveillance of MRSA, as characteristic genotypic traits correlated strongly with the epidemiology of MRSA within these settings. For example, the high virulence and transmissibility of CA-MRSA strains could be attributed to the presence of *lukS/lukF-PV pvl* genes, which were not commonly found in HA-MRSA strains (Hu *et al.*, 2015; Lakhundi and Zhang, 2018). Nowadays however, the lines have become increasingly blurred, with traditional HA-MRSA clones emerging outside of the healthcare environment into community settings (Fig. 1.1) (Choo, 2017).

Numerous CA-MRSA lineages have also emerged in healthcare settings in recent years. This includes major epidemic clones, such as the clonal complex (CC)8/sequence type (ST)8-MRSA-IV USA300 and the CC1/ST772-MRSA-V Bengal Bay clones which were originally described as CA-MRSA only (Blomfeldt *et al.*, 2017; Nimmo, 2012). These clones have now invaded hospitals and are widely reported as causal agents of nosocomial MRSA infections and outbreaks globally. The introduction of CA-MRSA clones into healthcare environments has resulted in greater clonal diversity and higher potential in transmission/virulence than HA-MRSA clones (Choo, 2017). It has been predicted that these clones will eventually replace many of the archetypal HA-MRSA clones within healthcare settings (Choo, 2017; Henderson and Nimmo, 2018). Kouyos *et al.*, 2013, utilised mathematical models to propose the likelihood of a balanced co-existence between HA- and CA-MRSA strains due to high rates of hospitalisation and discharges maintaining hospital–community interactions. Consequently, the conventional approach for categorising strains as either HA-MRSA or CA-MRSA based on molecular characteristics and virulence/resistance patterns are no longer fully accurate

(Choo, 2017; Otter and French, 2012). In practice, more accurate epidemiological analysis of MRSA should practice: (a) extensive scrutiny of details of acquisition and onset in either community or healthcare settings; and (b) employment of more comprehensive and informative genotyping techniques (Henderson and Nimmo, 2018; Otter and French, 2012).

1.7.1 Healthcare-associated MRSA

Healthcare-associated MRSA were often traditionally associated with nosocomial infections including infective endocarditis, surgical site infections, BSIs, pneumonia following invasive medical procedures, time in intensive care units or prolonged hospitalisation (Choo, 2017). Categorised based on virulence, multi-antibiotic resistance profiles and high incidence rates, HA-MRSA were always a major cause for concern in healthcare settings globally (Choo, 2017; Lindsay, 2013). The majority of HA-MRSA strains frequently reported belong to a few distinct lineages or CCs including CC5, CC8, CC22, CC30 or CC45 (Fig. 1.7) (Katayama *et al.*, 2005). While the CC5 and CC8 lineages are the most commonly observed nosocomial lineages worldwide, CC22 is predominantly present within Europe and Australia and CC30 within the UK (Chambers and Deleo, 2009). The CC45 lineage is mostly prevalent across Europe and the USA (Chambers and Deleo, 2009). These lineages typically carry larger SCC*mec* elements, particularly type I, II and III.

As a globally-relevant clonal complex, the dominant clones within the CC5-MRSA complex have been extensively investigated. Phylogenetic studies on these clones revealed high genetic diversity even amongst strains carrying the same SCC*mec* type circulating within the same country (Challagundla *et al.*, 2018; Geraci *et al.*, 2016). For example, a ST5/CC5 lineage, commonly referred to as the 'Paediatric Clone' is considered to be a clinically-relevant clone often recovered from neonates, adult patients and healthcare workers (HCWs) (Geraci *et al.*, 2016; McTavish *et al.*, 2019; Udo and Al-Sweih, 2017). Over the years, close proximity between MRSA-infected patients and HCWs alongside frequent direct contact between different healthcare personnel and also the hospital environment has been noted as an important factor in the successful dissemination of HA-MRSA (Cimolai, 2008). Analysis of studies and outbreak reports between 1980 and 2006 showed MRSA transmission likely occurred from HCWs to hospitalised patients in 93% (63/68) of cases (Albrich and Harbarth, 2008). Likewise, a study of the relationship between MRSA environmental contamination and patient

acquisition in a nine-bed intensive care unit (ICU) over a 14-month period showed that in 35.7% (20/56) of cases, MRSA isolated from patients were indistinguishable to those isolated from the environment (Hardy *et al.,* 2006). In most cases, national guidelines on the prevention and control of MRSA do not recommend routine MRSA screening of HCWs. Screening is typically only recommended following the identification of an infection cluster or outbreak (Department of Health, Ireland, 2013). The implementation of routine MRSA screening protocols for healthcare professionals and facility-wide environmental cleaning protocols should be considered as both have been shown to positively impact the management of HA-MRSA outbreaks in numerous instances (Ben-David *et al.,* 2008; Blok *et al.,* 2003; Garvey *et al.,* 2018; Watson *et al.,* 2016).

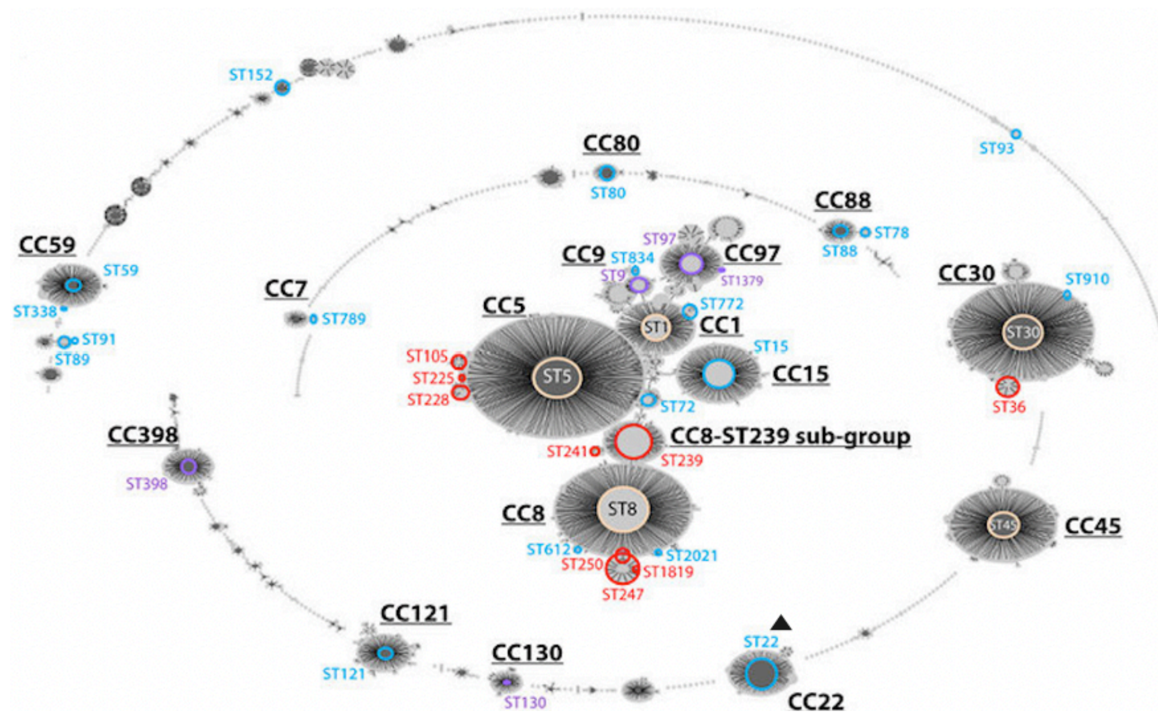


Figure 1.7. The global population structure of MRSA presented by eBURST analysis. The different sequence types (STs) and the corresponding cluster/clonal complex (CC) to which they belong are indicated. Circle size indicates the frequency of a particular ST within the associated CC group. Red circles represent HA-MRSA, blue circles represents CA-MRSA and purple circles represent LA-MRSA. The black triangle denotes the predominant HA-MRSA ST in Ireland currently. Adapted from Lakhundi and Zhang, 2018.

1.7.2 Community-associated MRSA

Community-associated MRSA were historically defined as MRSA responsible for infections in the community in healthy individuals without pre-disposing risk factors or recent contact with the healthcare environment (Otter and French, 2012). The prevalence of CA-MRSA has been widely investigated in the USA where the CC8/ST8-MRSA-IV USA300 clone emerged and became endemic before spreading to other parts of the world (Table 1.1) (Nimmo, 2012; Planet, 2017). USA300 carries the small SCC*mec* type IV element and harbours the *lukF/S-PV pvl* virulence genes typically associated with most community-associated clones (Fig. 1.8). Additionally, USA300 also harbours the arginine catabolic mobile element (ACME) gene cluster, which also facilitates the evasion of host immune responses, persistence on human skin and colonisation of mucosal membranes (Fig. 1.8) (Lepuschitz *et al.,* 2018). CC1/ST1-MRSA-IV USA400 is another prevalent CA-MRSA clone in the USA (Table 1.1). In Asia, the predominant CA-MRSA clone is the CC59/ST59-MRSA-IV, while in Europe, the CC30/ST30-MRSA-IV Southwest Pacific clone and CC80/ST80-MRSA-IV are widely prevalent CA-MRSA clones (Table 1.1) (Lepuschitz *et al.,* 2018).

CA-MRSA typically cause skin and soft tissue infections (SSTIs), but have also been linked with more severe, life-threatening conditions including osteomyelitis, sepsis and necrotizing pneumonia (Asghar, 2014; DeLeo *et al.,* 2010; Otto, 2012). The exceptional success of CA-MRSA strains is believed to be a result of the enhanced pathogenicity and transmissibility provided by the PVL cytotoxin, as well as the reduced fitness burden of harbouring less antimicrobial resistance genes and smaller SCC*mec* elements compared to the MDR profile and larger SCC*mec* elements carried by most HA-MRSA strains (Otto, 2012). Increased expression of core-genome encoded toxins, particularly PSMs and α-haemolysin have also been observed in many CA-MRSA clones (Fig. 1.8) (David and Daum, 2010; Rasigade *et al.,* 2013).

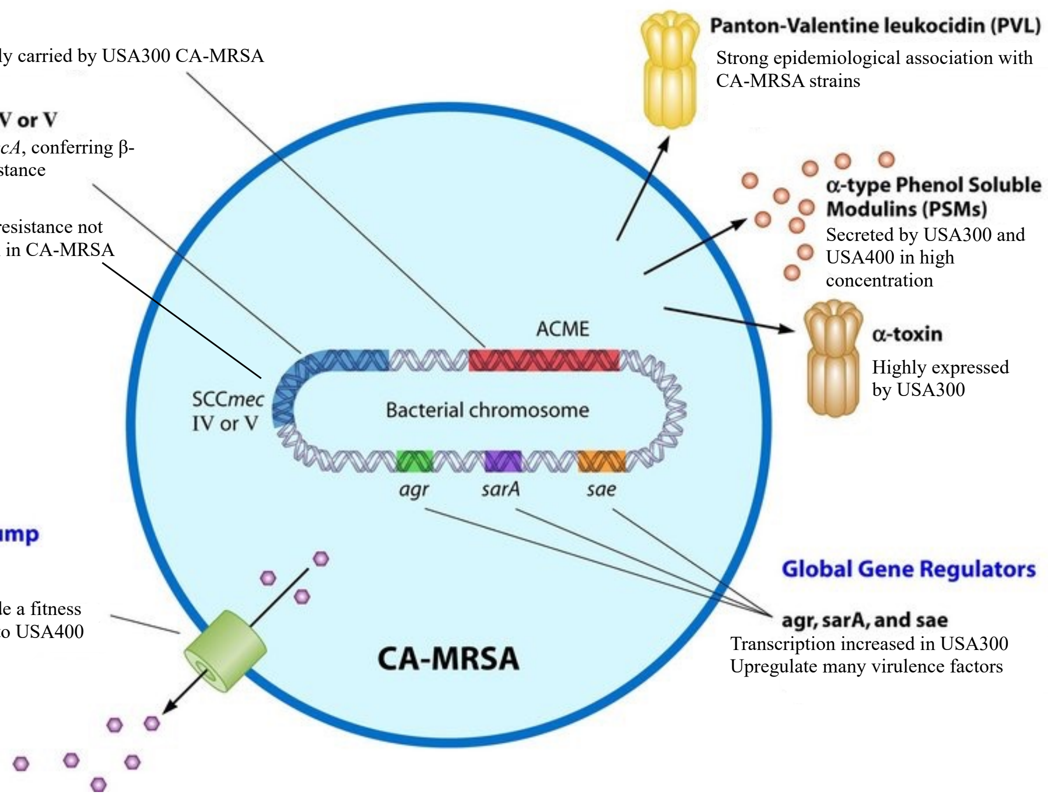


Figure 1.8. Schematic diagram of virulence factors typically linked with common CA-MRSA strains. Abbreviations: ACME, arginine catabolic mobile element; SCC*mec*, staphylococcal cassette chromosome *mec.* Adapted from David and Daum, 2010.

Table 1.1 Prominent community-associated (CA)-MRSA clones globally

Name	Lineage description	First reported	Year	Reference
Western Australia (WA)-1	PVL- ST1-MRSA-IV	Australia	1989	Udo *et al.,* 1993
Western Australia (WA)-2	PVL- CC88/ST78-MRSA-IV	Australia	1995	Coombs *et al.,* 2020
European (EDK-97) clone	PVL+ CC80/ST80-MRSA-IV	Europe: Denmark	1997	Stegger *et al.,* 2014
USA400/MW2	PVL+ CC1/ST1-MRSA-IV	USA	1999	Ammerlaan and Bonten, 2006
Queensland (Qld) clone	PVL+ ST93-MRSA-IVa	Australia	2000	Munckhof *et al.,* 2003
USA1100/South Western Pacific (SWP)	PVL+ CC30/ST30-MRSA-IV	South America: Uruguay	2002	Leme *et al.,* 2021
USA300	PVL+ CC8/ST8-MRSA-IV	USA	2003	Tenover *et al.* 2009
Bengal Bay	PVL+ ST772-MRSA-V	South Asia: India, Bangladesh	2004	Steinig *et al.,* 2019
Taiwan clone	PVL+ CC59/ST59-MRSA-V	East Asia: Taiwan	1997	Ward *et al.,* 2016
USA300-Latin American variant (LV)	PVL+ CC8/ST8-MRSA-IV	South America: Ecuador, Colombia	2005	Planet *et al.,* 2015
'African clone'	PVL- CC88/ST88-MRSA-IV	Sub-Saharan Africa: Ghana	1978	Wang *et al.,* 2022

Abbreviations: CC, clonal complex; PVL, Panton-Valentine leukocidin; +, positive; -, negative; ST, sequence type

1.7.3 Livestock-associated MRSA

Since its initial emergence in Belgium in the early 1970s amongst domestic cattle, the characterisation of LA-MRSA has become a developing area of research (Anjum *et al.,* 2019). Global epidemiological investigations on LA-MRSA outbreaks have identified livestock as domestic reservoirs for zoonotic transmission of MRSA into humans (Elstrøm *et al.,* 2019). Human carriage is now also strongly associated with direct prolonged exposure to infected farm animals, particularly in farm workers, their family members, veterinarians, and those working in abattoirs (Crombé *et al.,* 2013; Lakhundi and Zhang, 2018). A CC398 clone is the most commonly observed MRSA lineage associated with livestock globally (Fig. 1.7) (Lakhundi and Zhang, 2018). This clone originally emerged in pigs across Europe, but has now also been detected in other farm animals in Europe and North America and has since been linked to incidences of patient-to-patient transmission within hospital settings (Anjum *et al.,* 2019; Crombé *et al.,* 2013). CC398 isolates typically carry SCC*mec* types IVa or V, however other type IV variants, type III and non-typeable (NT) SCC*mec* elements have also been identified in pig strains (Butaye *et al.,* 2016). Other distinct lineages of MRSA reportedly associated with different livestock species include CC1, CC9 and CC130 (Fig. 1.7) (Elstrøm *et al.,* 2019; Lakhundi and Zhang, 2018). In CC130 for example, a *mecC*-positive MRSA strain has emerged as a novel human pathogen with zoonotic origins. These CC130-MRSA strains harbour the highly divergent SCC*mec* XI element on which the *mecC* gene is situated, and are predominantly associated with bovine sources (Lozano *et al.,* 2020; Shore *et al.,* 2011). Although this ST130-MRSA-XI lineage has also been identified in small infected/colonised wild animals including hedgehogs (Monecke *et al.,* 2013a). Interestingly, the samples taken from these wild animals were from 2003 (the earliest detection of this strain), long before identical isolates were also recovered in humans, suggesting that ST130-MRSA-XI carrying *mecC* is a zoonotic lineage which originally emerged in small wild animals, before spreading to bovine hosts and then to humans (Lozano *et al.,* 2020; Monecke *et al.,* 2013a). Although LA-MRSA has contributed very little to the healthcare burden of MRSA so far, with infection/transmissions still relatively low and human colonisation mostly being asymptomatic and transient, continuous widespread misuse of antimicrobial agents within agricultural and veterinary settings could eventually exacerbate this burden (Butaye *et al.,* 2016; Sharma *et al.,* 2016).

1.8 MRSA in Ireland

For several decades, the burden of MRSA has had a significant impact on Irish hospitals and the community, playing a major role in the prevalence of infectious diseases and responsible for widespread morbidity and mortality in Ireland (Looney *et al.,* 2017). Currently, over 1 in 10 cases (10.6%) of all invasive *S. aureus* infections in Ireland are associated with MRSA (EARS-Net, 2021). When MRSA first became endemic in Irish hospitals in the early 1970s, the majority of nosocomial cases reported were predominantly surgical wound infections and traumatic skin lesions (HSE, 2005). Nowadays, MRSA contributes significantly to the incidence of bloodstream infections, osteomyelitis, infective endocarditis and necrotizing pneumonia. By the mid-1990s, MRSA began to emerge outside hospitals in Ireland, particularly within nursing homes and other healthcare-related settings. A study carried out in Dublin, Ireland during this period revealed that 8.6% of residents within six different nursing homes were carriers of MRSA (HSE, 2005). Additionally, 24% of environmental samples taken from these nursing homes were also positive for MRSA (HSE, 2005). Shortly after this, reports of community-associated MRSA infections in individuals within the general population with no underlying risk factors or close contact with hospitalised patients began to appear. Recent findings indicate that approximately 12% of all MRSA isolates now recovered in Ireland are community-associated, with the majority of these manifesting primarily as skin and soft tissue infections (SSTIs) (HSE, 2005).

As a result of extensive surveillance on both HA- and CA-MRSA in Ireland over the years, the epidemiology of prevalent clonal types, major shifts in patterns of circulation and timelines of predominance are all well-documented. The ST250-MRSA-I/I-*pls* clone was prevalent in Irish hospitals in the 1970s and early 1980s, followed by ST239-MRSA-III-pI258/Tn*554* in the mid-to-late 1980s, and then ST8-MRSA-II in the early-1990s (Kinnevey *et al.,* 2014; Shore *et al.,* 2005). This continued into the late 1990s before the clone was displaced by ST36-MRSA-II and then ST22-MRSA-IV shortly after in 2002 (Kinnevey *et al.,* 2014). Since then, this PVL-negative ST22-MRSA-IV clone (often referred to as the EMRSA-15 clone) which often results in invasive nosocomial bloodstream infections has maintained its prevalence in hospitals in Ireland and in many other countries across Europe (Broderick *et al.,* 2021). The clone currently accounts for approximately 70–80% of all MRSA-BSI isolates recovered in Irish hospitals (66.6% in 2021) (NMRSARL, 2020; NMRSARL, 2021). The increased diversity (greater

resistance profiles and virulence gene carriage) in MRSA clones being introduced into Ireland in recent times suggests that displacement of this highly successful HA-MRSA ST22 clone could occur at any time (NMRSARL, 2020). In relation to predominant CA-MRSA clones in Ireland, ST8-MRSA-IV and ST30-MRSA-IV have been the most notable, resulting from the importation of novel strains and lineages over the years (Brennan *et al.,* 2012).

1.8.1 Surveillance

Surveillance of antimicrobial resistant pathogens in Ireland including MRSA is carried out as part of an international collaboration with the European Centre for Disease Prevention and Control (ECDC). Ireland contributes to this collection and reporting of accurate data on antimicrobial resistance (AMR) through the publicly-funded ECDC European Antimicrobial Resistance Surveillance Network (EARS-Net). Since its establishment in 2002, the Irish National MRSA Reference Laboratory (NMRSARL) has acted as the national surveillance laboratory for the EARS-Net project (NMRSARL, 2021). Based in St. James's Hospital in Dublin, all relevant MRSA samples from hospitals and community health centres are referred to the NMRSARL for identification and genotyping to confirm suspected outbreaks and transmission. The carriage of novel antimicrobial resistance mechanisms and the *pvl lukS/F-PV* virulence genes by CA- and HA-MRSA isolates is also monitored by the reference laboratory.

1.8.2 Prevalence

According to a recent report published by EARs-Net, out of 1,232 *S. aureus* isolates received by the NMRSARL in 2021 as part of the EARs-Net surveillance project, only 130 of these were MRSA (EARS-Net, 2021). This indicates a 10.6% decrease in the number of MRSA BSIs in Ireland between 2017–2021 (Fig. 1.9) (EARS-Net, 2021). The Irish Department of Health also reported on the gradual decrease in MRSA BSIs since the alarming peak in 2006 when the incidence of MRSA BSIs reached 42% (Department of Health, Ireland, 2018). This figure declined to 22.8% in 2012, 16.1% in 2017 and then 10.5% in 2021(HSE HPSC, 2019; Kinnevey *et al.,* 2014). The epidemic PVL-negative MRSA ST22-IV clone has remained the predominant cause of BSIs in Ireland. Despite this decline in the number of reported MRSA BSIs over this 15 year period, Ireland still ranked mid-level (14th out of 29 EARS-Net countries) in terms of MRSA-BSI

distribution in 2021, indicating that MRSA should still be considered a major threat to public health (Fig. 1.9) (HSE HPSC, 2019).

The proportion of PVL-positive MRSA clones has steadily increased since 2002 (O'Connell and Brennan, 2014). These pathogens were mostly responsible for SSTIs and in some cases, invasive illnesses. The number of PVL-positive MRSA submitted to the NMRSARL increased from 0.2% in 2002 to 1.4% in 2006, and by 2011, was at 8.8% (NMRSARL, 2018; Shore *et al.,* 2014). In 2022, 16.8% of non-BSI MRSA isolates received by the NMRSARL were PVL-positive (NMRSARL, 2021). As in previous years, the predominant sequence types for these PVL-positive isolates were ST5, ST8 and ST30, which all typically carry type IV or V SCC*mec* elements (NMRSARL, 2014; NMRSARL, 2018; Shore *et al.,* 2014). These PVL-positive MRSA strains caused outbreaks within nosocomial and community settings in Ireland, further confirming the unreliability of using expression of PVL toxin as an indicator of MRSA’s community association. Most recently, an assessment of recent trends and incidence of MRSA in Ireland by the NMRSARL revealed the presence of a traditional LA-MRSA lineage (ST398) within the community (NMRSARL, 2021). This PVL-positive strain is now frequently being associated with colonisation and infection of individuals with epidemiological links to Southeast Asia.

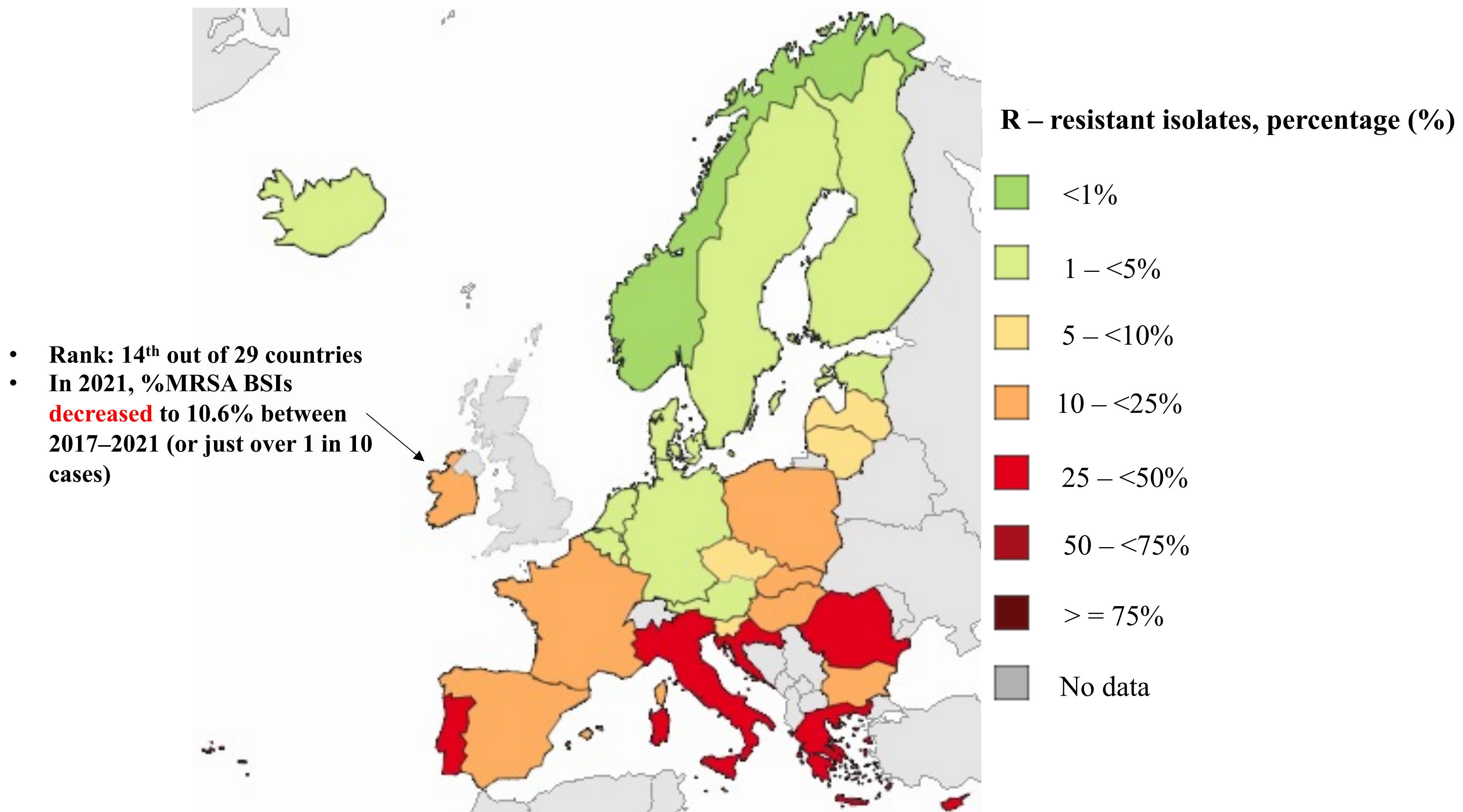


Figure 1.9. Distribution and prevalence of MRSA bloodstream infections (BSIs) in EARS-Net countries in 2021. Adapted from EARS-Net, 2021.

Additionally, increased incidence of the PVL-positive MDR clone (ST772-MRSA-V), commonly referred to as the 'Bengal Bay clone' has been observed in Ireland (NMRSARL, 2021). This CA-MRSA clone is frequently associated with skin/soft tissue infections and has led to several outbreaks within Irish hospitals in recent years. Constant movement and transfer of patients, HCWs and visitors between different healthcare environments could potentially be contributing to the introduction of PVL-positive CA-MRSA into healthcare settings (Earls *et al.,* 2017). Additionally, socioeconomic factors which primarily include movement of HCWs from abroad, increased international travel and recent displacement followed by subsequent migration of those fleeing conflict into Ireland also contribute to the importation of novel MRSA strains into Ireland (Choo, 2017; Otter and French, 2012; Shore *et al.,* 2014).

1.8.3 Infection prevention control measures in Ireland

Currently, treatment of confirmed MRSA infections is dependent on infection type (HSE, 2005). Doxycycline from the tetracycline antibiotic class or co-trimoxazole are recommended for treating non-severe cases except when infections occur in those who are pregnant or in children under the age of twelve. Glycopeptides such as vancomycin are recommended and limited for the treatment of severe cases where use is appropriate. Prolonged use of glycopeptide therapy is not common practice due to the association between extended use and selection of glycopeptide resistance. Linezolid, daptomycin or clindamycin are the alternative treatment options available for MRSA infections.

In MRSA-positive patients, decolonisation is often only considered if the patient is at risk of developing an infection or if persistent MRSA transmission has been noted within a specific hospital unit (HSE, 2005). The most effective MRSA decolonisation therapy currently available in Ireland is nasal and body decolonisation typically performed using 2% topical nasal mupirocin and 4% chlorhexidine or 7.5% povidone-iodine (HSE, 2005). This decolonisation approach focuses primarily on nasal carriage and completely ignores patients who present exclusively with oral colonisation. A recent study carried out within inpatient wards of a tertiary referral hospital in Ireland over a two-year period screened for MRSA and MSSA in patients and HCWs using nasal swabs and oral rinses (Kearney *et al*., 2020). The outcome of the study showed that the oral cavity is a significant reservoir for *S. aureus* as a significant proportion of those who underwent screening were only colonised orally. Decolonisation practices should include routine oral screening,

especially in those who experience recurrent infections or those considered to be recurrent MRSA carriers where decolonisation attempts have repeatedly failed.

According to current Irish national clinical guidelines, targeted screening is more appropriate as opposed to universal screening of all patients (HSE, 2005). Routine screening of HCWs is also not mandatory, unless HCWs are directly linked by epidemiological evidence to a particular cluster of MRSA infections. Typically, only patients exhibiting pre-defined MRSA risk factors are screened upon admission into hospitals. This includes patients re-admitted after previously being MRSA-positive, patients who were transferred from another hospital/healthcare facility and those who were in-patients within the last six months. Other risk factors include patients admitted with skin conditions or non-intact skin and those undergoing high/medium risk surgeries or renal dialysis. Patients in the intensive care unit (ICU) or special care baby unit (SCBU) are also screened upon admission, with the latter undergoing routine screening weekly thereafter. Routine screening of patients is also commonly only practiced in cases of outbreaks or confirmed transmission within a specific hospital ward.

In relation to CA-MRSA, screening to detect asymptomatic carriage between close contacts is currently not recommended practice in Ireland unless advised by a clinician (HSE, 2005). Screening is typically only offered when ongoing infections are occurring within the same household or closely-associated cohort e.g., prison, nursing homes or military camps. In practice, these recommendations may be inadequate as recent findings within a large acute hospital in Dublin, Ireland highlighted widespread transmission of MRSA and MSSA (screening for carriage of MSSA is even less common) from HCW-to-patients, HCW-to-HCW, patient-to-patient and environmental contamination by HCW/patient, all under non-outbreak conditions over extended periods of time (Kinnevey *et al.,* 2021; Kinnevey *et al.,* 2022). Comprehensive investigations into the epidemiology of MRSA are fundamental to improving current IPC measures and reducing the human and financial impact of this pathogen. The advent of WGS provides a highly discriminatory tool with unprecedented resolution for effectively monitoring MRSA and better informing IPC strategies.

1.9 Project aims

The epidemiology of MRSA in Ireland is continuously evolving, with numerous distinct CA-MRSA clones emerging and disseminating widely across hospitals and community settings in recent years. Ongoing surveillance is critical in understanding MRSA epidemiology and controlling the spread of emerging clones. Whole-genome sequencing provides a suitable high-resolution tool for accurately tracking spread, monitoring transmission and characterising novel strains (Earls *et al.,* 2018; Earls *et al.,* 2017). Accordingly, the goal of this project was to expand on the currently limited knowledge of CA-MRSA in Irish hospitals using WGS.

- The first research chapter (Chapter 3) of this project aimed to employ WGS to investigate multiple distinct PVL-positive CA-MRSA outbreaks in numerous Irish hospitals. This study also aimed to characterise the CA-MRSA population currently circulating in Irish hospitals. This primarily focused on CA-MRSA associated with maternity patients, as these are typically healthy individuals with minimal MRSA risk factors, including limited exposure to the healthcare environment.

- Following on from the first part of this project where a novel PVL-positive CA-MRSA ST5-MRSA-IVc clone was identified and associated with a protracted maternity unit outbreak in Ireland, the second research chapter (Chapter 4) aimed to investigate this emerging clone using WGS. Similar isolates had recently been described in Sri Lanka, Australia and the UK. A WGS-based phylogenetic framework was constructed to assess the widespread international dissemination and diversity of this PVL-positive CA-MRSA clone across twelve different countries over a 17-year period.

- In Chapter 4 of this project, the dissemination of the novel PVL-positive ST5-MRSA-IVc 'Sri Lankan' clone was described. The final research chapter (Chapter 5) of this project aimed to further characterise this emerging clone by using WGS to investigate the PVL-encoding bacteriophage lysogenised into the bacterial genome. The distinct PVL-encoding bacteriophage remnant associated with the PVL-positive Sri Lankan clone had previously not been described in the

literature. In-depth characterisation of this remnant could provide insights into the successful dissemination of the Sri Lankan clone, its evolutionary origins and its relationship to other MRSA clones.

Chapter 2

General Materials and Methods

2.1 Bacterial Isolates

A total of 662 *S. aureus* isolates were investigated in the present study. This included isolates recovered in (i) Ireland between 2011–2022 (PVL-positive MRSA [*N*=109], PVL-positive MSSA [*N*=1], PVL-negative MRSA [*N*=273]) and (ii) isolates recovered internationally between 1998–2021 (PVL-positive MRSA [*N*=253], PVL-positive MSSA [*N*=15], PVL-negative MRSA [*N*=11]). Table 2.1 provides a breakdown of all isolates investigated.

2.2 General Microbiological Methods

2.2.1 Microbial culture and long-term storage

For long-term storage, all isolates were individually preserved in cryogenic bead vials using the Protect Bacterial Preservation System (Technical Services Consultants Ltd., Heywood, UK) and stored at -80°C. Routine culture of isolates was performed by aseptically streaking a single bead onto Columbia Blood Agar (CBA; Fannin Ltd., Dublin, Ireland), followed by incubation at 37°C for 18 h in a static incubator (Gallenkamp, Leicester, UK).

2.2.2 Chemicals, water and reagents

All chemicals used were of analytical grade or molecular biology grade and were purchased from Merck (Sigma-Aldrich Ireland Limited, Arklow, Ireland) or the Promega Corporation (Madison, Wisconsin, USA), unless otherwise stated. Preparation of buffers and other chemical solutions was performed using molecular grade water generated by the Millipore Milli-Q® IQ 7003 Pure and Ultrapure Water Purification System (Merck, Millipore Ireland, Co. Cork, Ireland). For preparation of agarose gels and gel electrophoresis buffers, 5× stock solution of Tris-borate/EDTA buffer (TBE) was prepared using 0.45 M Tris base, 0.45 M boric acid and 0.01 M EDTA with a pH of 8. The stock was diluted to a working concentration of 0.5× prior to use. Enzymes, buffers and deoxynucleotide triphosphates (dNTPs) were purchased from Promega. All oligonucleotide primers used were purchased from Merck.

Table 2.1 Details of all 662 *S. aureus* isolates investigated in the present study

Chapter	Purpose of the study	Number of isolates		Years of isolation	Source of isolates
		MRSA	MSSA		
3	CA-MRSA in Ireland: Investigating emerging lineages and distinct outbreaks of PVL-positive CA-MRSA in hospital settings by WGS	358	14	2011–2022	Australia, China, Egypt, Germany, India, Ireland, Namibia, Nepal, Saudi Arabia, UAE, UK and USA
4	An emerging Panton-Valentine leukocidin (PVL)-positive CC5-MRSA-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by WGS	285[a]	0	2003–2022	Algeria, Australia, Czech Republic, Denmark, Germany, Ireland, Kuwait, Norway, Saudi Arabia, Senegal, Slovakia, Sri-Lanka, Sweden, UAE and UK
5	Characterisation of a 9.6 kb Panton-Valentine leukocidin (PVL)-encoding bacteriophage remnant harboured by a CC5- MRSA-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by WGS	283[b]	2	2003–2022	Algeria, Australia, Czech Republic, Denmark, Germany, Ireland, Kuwait, Luxembourg, Norway, Saudi Arabia, Senegal, Slovakia, Sri-Lanka, Sweden, UAE and UK

[a] Includes 14 of the isolates from Chapter 3 and 46 previously described isolates (McTavish *et al*., 2019)

[b] Includes all 266 isolates from Chapter 4

Abbreviations: WGS, whole genome sequencing; CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus.*

2.2.3 Liquid handling

Pipetting and dispensing of microvolumes of liquid was performed using one of the single channel Gilson pipettes (P2L, P20L, P200L, P1000L Gilson™ PIPETMAN™ Classic 4-Pipette Kit) (Gilson, Wisconsin, USA) which cover volume transfers between 0.2–1000 µl. For repetitive pipetting of microvolumes of liquid (20–200 µl), transfer was performed using either the 8-channel or 12-channel ErgoOne® multichannel pipette (Starlab Group, Hamburg, Germany). Disposable pipette tips at 20, 200 and 1000 µl were used with the pipettes (Starlab Group). For dispensing larger volumes (1–25 ml), the PIPETBOY acu 2 (Integra Biosciences, Berkshire, UK) pipette controller was used.

2.3 Isolate molecular characterisation

Identification, molecular typing analyses of all isolates recovered in Ireland was undertaken at the National MRSA Reference Laboratory (NMRSARL), St. James's Hospital, Dublin (Table 2.1).

2.3.1 Identification of *S. aureus* isolates

Isolates were confirmed as *S. aureus* at the NMRSARL using the tube coagulase test which detects extracellular staphylocoagulase as previously described (Rossney *et al*., 1990). Isolates were also tested using the Pastorex™ Staph-Plus Latex Agglutination Test (Bio-Rad, Marnes la Coquette, France), which detects fibrinogen affinity antigen (clumping factor), protein A, and capsular polysaccharides produced by *S. aureus* according to the manufacturer's instructions.

2.3.2 Identification of MRSA isolates

Methicillin resistance was determined with 30 µg cefoxitin disks (Oxoid Ltd., Basingstoke, UK) using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology and interpretive criteria (EUCAST, 2023) ((EUCAST), 2023). Methicillin resistance-encoding genes were detected in isolates by multiplex PCR targeting the *mecA* and *mecC* genes using *mec* gene-specific oligonucleotide primers (NMRSARL, 2018; Oliveira and de Lencastre, 2002; Shore *et al.,* 2011). The 20 µl PCR reaction volumes contained the relevant oligonucleotide primers (Merck), 1.5 mM $MgCl_2$, 5× Green GoTaq Flexi buffer and 2.5 U GoTaq DNA polymerase (Promega). The following thermal cycling conditions were used: 94°C for 2 min, followed by 35

cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min.

2.3.3 *spa* typing and identification of *pvl* genes

Isolates underwent conventional *spa* typing (NMRSARL, 2018). The *spa* typing process involved genomic DNA extraction using the InstaGene matrix solution according to manufacturer's instructions (BioRad, München, Germany), followed by PCR amplification of the polymorphic X region in the staphylococcal protein A (*spa*) gene. Primers, protocols and PCR conditions used have been described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; www.seqnet.org/). Purification of PCR products was carried out using the GenElute PCR Clean-Up Kit (Merck) and commercial Sanger-based sequencing was performed using Eurofins Genomics DNA sequencing services (Eurofins, Konstanz, Germany). Detection of the Panton-Valentine Leukocidin (PVL)-encoding genes, *lukF/S-PV* was performed by multiplex PCR to obtain co-amplification of the two genes as previously described (Lina *et al.,* 1999; NMRSARL, 2018). The PCR assays contained oligonucleotide primers (Merck), sample template, dNTPs, $MgCl_2$, 5× Green GoTaq Flexi buffer and GoTaq DNA polymerase (Promega).

2.3.4 Antimicrobial susceptibility testing

Isolates underwent antimicrobial susceptibility testing against a panel of 18 antimicrobial agents using EUCAST and Clinical Laboratory Standards Institute (CLSI) approved methodology and interpretive criteria as previously described (CLSI, 2020; EUCAST, 2023). Where interpretive criteria are not available from EUCAST or CLSI, criteria developed at the NMRSARL was used (NMRSARL, 2014; Rossney *et al.,* 2007). MDR was defined as resistance to ≥3 separate classes of clinically relevant antibiotics. All 23 antimicrobial agents tested are listed in Table 2.2, alongside disk concentrations and the most up-to-date relevant clinical breakpoints (CLSI, 2020; McManus *et al.,* 2015).

Table 2.2. Antimicrobial agents and clinical breakpoints used for antimicrobial susceptibility testing

Antimicrobial agent	Disk concentration (µg/disk)	Zone diameter breakpoints (mm)[a]			Reference for interpretive criteria
		S (≥)	I[b]	R(≤)	
Amikacin (Ak)	30	15	None	15	(EUCAST, 2023)
Ampicillin (Ap)	10	29	None	28	(CLSI, 2020)
Chloramphenicol (Cl)	30	18	None	18	(EUCAST, 2022)[c]
Ciprofloxacin (Cp)	5	50	None	21	(EUCAST, 2023)
Erythromycin (Er)	15	21	None	21	(EUCAST, 2023)
Fusidic acid (Fd)	10	24	None	24	(EUCAST, 2023)
Gentamicin (Gn)	10	18	None	18	(EUCAST, 2023)
Kanamycin (Kn)	30	18	14–17	13	(CLSI, 2020)
Lincomycin (Ln)	2	17	15–16	14	(Rossney *et al*., 2007)
Linezolid (Lz)	10	21	None	21	(EUCAST, 2023)
Mupirocin (Mp)	200	30	16–29	15	(Rossney *et al*., 2007)
Neomycin (Nm)	30	18	16-17	15	(Rossney *et al*., 2007)
Rifampicin (Rf)	5	26	None	26	(EUCAST, 2023)
Streptomycin (St)	25	16	14–15	13	(Rossney *et al*., 2007)
Tetracycline (Te)	30	22	None	22	(EUCAST, 2023)
Tobramycin (Tb)	10	18	None	18	(EUCAST, 2023)
Trimethoprim (Tp)	5	14	None	14	(EUCAST, 2023)
Vancomycin (Vn)	30	15	None	14	(CLSI, 2020)

[a] Zones of growth inhibition around each antibiotic disk were recorded in mm and interpreted as resistant (R), intermediate (I) and susceptible (S), according to the guidelines referenced (CLSI, 2020; EUCAST, 2023; Rossney *et al.,* 2007).

[b] None, no intermediate breakpoint indicated for this antimicrobial agent in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020; EUCAST, 2023).

[c] According to the latest EUCAST report (2023), the clinical efficacy of chloramphenicol has been questioned and breakpoints are currently under review (30th March 2023).

2.4 Genomic DNA Isolation

2.4.1 Qiagen DNeasy® Blood and Tissue Kit extraction

For genomic DNA extractions, all isolates were re-activated from the -80°C stock onto fresh CBA plates. This was done by removing a single bead using sterile forceps from the cryogenic bead vial, inoculating this bead onto a plate using a sterile inoculating loop and incubating this plate overnight at 37°C in a static incubator. Following 18 h incubation, a single colony from the reactivated culture was then lawned onto half of a fresh CBA plate and incubated overnight at 37°C in a static incubator. Genomic DNA extractions through enzymatic lysis of the bacterial cells were performed using Inter-Array lysis buffer and lysis enhancer (Iter-Array fzmb GmbH, Bad Langensalza, Germany) and the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, West Sussex, UK) in accordance with the manufacturer's instructions. A 1 µl inoculating loop of the lawned culture was added into 200 µl of lysis buffer/lysis enhancer in a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) and incubated at 37°C for 3 h in a shaking thermomixer (Eppendorf ThermoMixer® C). Following lysis, 25 µl of Proteinase K and 200 µl of AL buffer (both supplied with the Qiagen kit) was added to the mixture to digest proteins in the lysate. Samples were then incubated at 70°C for 30 min in a heating block (Grant Instruments Cambridge Ltd., Shepreth, UK). Following incubation, 200 µl of ice cold 100% (v/v) EtOH was added to the mixture to increase yield of DNA and aid precipitation. DNA isolation was performed according to manufacturer's instructions using DNeasy Mini spin columns with silica membranes that bind DNA, collection tubes and 500 µl AW1/AW2 wash buffers (all supplied with the Qiagen kit). Extracted DNA was eluted in 100 µl of sterile, prewarmed molecular-grade water in fresh Eppendorf tubes and then concentrated by heating to 70°C for 30 min with the lids of the tubes open. Samples were stored at 4°C (short-term) or at -20°C (long-term).

2.4.2 Quality assurance and concentration of extracted DNA

The concentration and purity of extracted nucleic acid was measured by UV absorbance-based quantification on the NanoDrop spectrophotometer 2000c (Fisher Scientific™, Massachusetts, USA). To confirm purity of each DNA sample, 260/280 nm and 260/230 nm absorbance ratios of approximately 1.8–2.0 and 2.0–2.2, respectively, were deemed acceptable. For samples undergoing WGS, DNA concentration was further confirmed using the Qubit Fluorometer 3.0 (Invitrogen/Fisher Scientific™, Massachusetts, USA).

2.4.3 Gel electrophoresis

To further confirm the integrity and quality of extracted genomic DNA, samples were visualised by gel electrophoresis using 1% (w/v) agarose gels. Gels were prepared by dissolving 4 g of agarose powder (Merck) in 400 ml 0.5× TBE buffer (see Section 2.2.2). Agarose gels were casted in 8 x 10 cm gel trays with 1.5 mm 20-well combs (Genesee Scientific, San Diego, California, USA). DNA loading dye was purchased from Promega and DNA molecular size markers from Bioline (Singapore City, Singapore). Gel-Red nucleic acid stain (Biotium, Fremont California, USA) for fluorescent staining of the DNA was added to the melted agarose gel at a final concentration of 1×. Gel electrophoresis was performed in a Galileo Bioscience gel box (Cambridge, Massachusetts, USA) using a Consort™ Power Supply EV222 (B-2300 Turnhout, Belgium) set at 90 V and 80 mA for 1 h. Following electrophoresis, gels were visualised under ultraviolet light in the Alpha Innotech Transilluminator AVT26U (Protein Simple, California, USA) and the AlphaImager Mini software (Protein Simple). The gels were photographed and captured images were printed on a Mitsubishi P93DW printer (Sant del Vallés, Barcelona, Spain).

2.5 Whole-genome sequencing

2.5.1 Second generation sequencing (Illumina)

Genomic DNA extraction was performed using the DNeasy® Blood and Tissue Kit (Qiagen) as described in Section 2.4. All isolate DNA samples underwent sequencing preparation using the Illumina® DNA Prep (M) Tagmentation Kit (Illumina, Eindhoven, The Netherlands) (Fig. 2.1). The libraries were scaled to yield a minimum of 70× coverage per isolate.

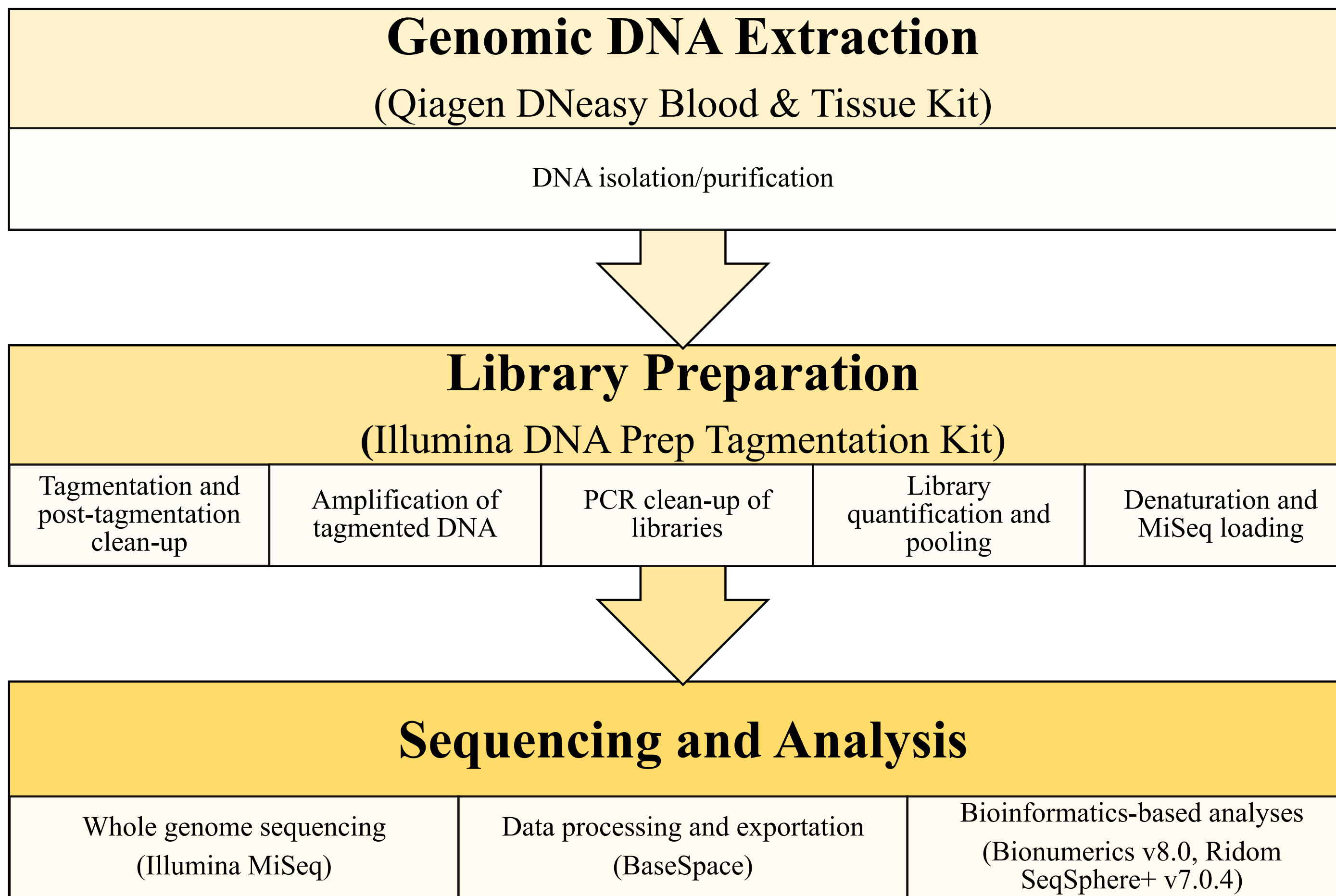


Figure 2.1. Simplified workflow of the short-read whole genome sequencing process employed for the present study

2.5.1.1 Tagmentation and post-tagmentation clean-up

Tagmentation of samples was performed using 15 µl of 250–300 ng diluted genomic DNA, 5 µl of Tagmentation Buffer and 5 µl of Bead-Linked Transposome (both supplied with the Illumina kit) mixed in 0.2 ml PCR tubes (Fisher Scientific™, Dublin, Ireland). The mixture was incubated at 55°C for 15 min in a PCR thermal cycler (Biometra TOne, Thistle Scientific Ltd., Glasgow, UK). To stop tagmentation, each sample was resuspended in 5 µl of Tagment Stop Buffer (Illumina) and incubated again at 37°C for 15 min. Following incubation, the content of each tube was transferred into separate wells of a 96-well PCR plate (Fisher Scientific). The plate was placed onto a 96-well plate magnet (Fisher Scientific) for 3 min. When the supernatant separated from the beads, the supernatant was discarded and the plate was removed from the magnet. Samples were washed by resuspending in 100 µl of Tagment Wash Buffer (Illumina), placing the plate back on the magnet and discarding the supernatant once clear. The washes were repeated three times.

2.5.1.2 Amplification of tagmented DNA

Immediately following post-tagmentation clean-up, samples were resuspended in a master mix of 10 µl Enhanced PCR Mix (Illumina) and 10 µl nuclease free water. Samples were transferred back into fresh 0.2 ml PCR tubes and 5 µl of the dual index adapter primer mix (Illumina Nextera DNA CD Indexes (96 Indexes, 96 Samples)) was added from the 96-well plate. A unique index adapter well was used for each sample to be loaded onto the same run. Sample libraries underwent amplification on the PCR thermal cycler using the following conditions: 68°C for 3 min, 98°C for 3 min, 5/6 cycles of 98°C for 45 s, 62°C for 30 s, and 68°C for 2 min, then 68°C for one min, followed by a 10°C hold.

2.5.1.3 PCR clean-up of libraries

Following amplification, the content of each tube was transferred into separate wells of a 96-well PCR plate and the plate was placed on the magnet for 5 min. Once clear, 22.5 µl of the supernatant was transferred into separate wells of a 96-well Midi plate (Abgene/Fisher Scientific™, New Hampshire, USA) alongside a master mix of 22.5 µl Sample Purification Beads (Illumina) and 20 µl nuclease free water for PCR clean-up of the amplified libraries. Samples were thoroughly mixed, incubated at room temperature for 5 min, then placed onto the magnet for a further 5 min. Once clear, 62.5 µl of the

supernatant was transferred into fresh wells of the Midi plate with 7.5 µl of undiluted Sample Purification Beads (Illumina). Samples were thoroughly mixed, incubated at room temperature for 5 min, then placed back onto the magnet for a further 5 min. With the plate still on the magnet, the supernatant was discarded and samples were covered with 100 µl of freshly prepared 80% ethanol (v/v) and incubated for 30 s. The ethanol wash was repeated twice and the samples were left to air dry for 5 min. The plate was then removed from the magnet, and samples were resuspended in 17 µl of Resuspension Buffer (Illumina). Samples were incubated at room temperature for 2 min before being placed back onto the magnet for a further 2 min. Once clear, 15 µl of the supernatant was transferred into fresh 0.2 ml PCR tubes. Samples were stored at -20°C for up to 30 days.

2.5.1.4 Library quantification and pooling

The concentration of each DNA sample was measured using the Qubit Fluorometer 3.0 (Invitrogen), according to manufacturer's instructions. Samples exhibiting concentrations <4 ng/µl were excluded and library preparation was repeated on these samples. For the remaining samples, DNA library concentrations were adjusted to 4 nM, and 5 µl of each sample (up to 40 and 60 samples for a MiSeq Reagent Kit v2 or v3 cartridge (Illumina), respectively) was pooled into one Eppendorf tube. The final concentration of the pooled library was confirmed using the Qubit Fluorometer 3.0. A concentration of approximately 2 ng/µl was deemed appropriate, as per Illumina protocols.

2.5.1.5 Denaturation and MiSeq loading

In a clean Eppendorf tube, 5 µl of freshly diluted 0.2 N NaOH was added to 5 µl of the 4 nM pool and incubated at room temperature for 5 min to allow for denaturation of the pooled library. The denatured pool was then diluted to a concentration of 12 pM using prechilled HT1 Hybridisation Buffer (Illumina). The PhiX (Illumina) adapter-ligated library used as a control for Illumina sequence runs was also diluted to 12 pM using the HT1 buffer. Immediately before loading the library, 6 µl of the PhiX control library was added to 594 µl of the denatured/diluted DNA library. To ensure full denaturation and separation of the DNA strands of the library, the sample was incubated at 96°C for 2 min on a heating block (Grant Instruments), followed by 5 min incubation on ice. A 500-cycle v2 (or a 600-cycle MiSeq Reagent v3 kit to extend read lengths from 2 × 250 bp paired-

end reads up to 2 × 300 bp and also double the amount of output per flow cell) MiSeq paired-end sequencing kit (Illumina) was used to sequence the pooled normalised DNA libraries on an Illumina MiSeq instrument according to manufacturer's instructions.

2.5.1.6 Sequence data processing and exportation

The quality of each sequencing run could be visualised on the Illumina MiSeq system through the cluster density and quality score (Q30) assessments according to the manufacturer's instructions. The cluster density value was used to predict data quality and yield of run with the optimal raw cluster density of v2 Reagents set at 1000-1200 K/mm^2 and v3 Reagents set at 1200–1400 K/mm^2, as per manufacturer's guidelines. The error rates in base calling were accessed by the quality score whereby a score of 30 represented an error rate of 1 in 1000 with the corresponding accuracy of the base calls at 99.9%. On completion of the sequencing run, the Illumina MiSeq system performed adapter trimming to demultiplex the sequence reads into separate FASTQ files. The generated FASTQ files were then exported from the MiSeq via the Illumina BaseSpace cloud (Fig. 2.2) (https://basespace.illumina.com/).

2.6 Bioinformatic Analyses

2.6.1 Genome assembly (*de novo* assembly)

For short-read sequencing, the resulting FASTQ files were submitted directly into the BioNumerics calculation engine (BioNumerics v8.0; Applied Maths, Sint-Martens-Latem, Belgium). The BioNumerics incorporated SPAdes assembly tool v3.7.1 (Applied Maths) was used to perform *de novo* assembly (Fig. 2.2). Quality of assembled reads was assessed using the quality statistics in-built tool within BioNumerics v8.0 (Applied Maths). Sequence reads were assessed based on the average read quality (average score >30) as per software developer guidelines. The average quality of the reads retained after trimming were expected to be higher than that of the raw sequence reads as bad quality reads had been filtered and trimmed out. The *de novo* assemblies were assessed based on the length of the median contig (N50 >100,000). The number of contigs observed in each isolate were expected to be <400. The short-read FASTQ datasets were also imported into the VELVET genome assembly tool (v1.2.10) incorporated within Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) (Fig. 2.2). Quality filtering was

undertaken with SeqSphere+ (Ridom) and trimmed reads were *de novo* assembled using default settings.

2.6.2 Genotyping and SCC*mec* subtyping

Antimicrobial resistance profiles, virulence-associated genes, *spa* types and traditional MLST were determined *in silico* using appropriate SeqSphere+ (Ridom GmbH) task template tools (Bletz *et al*., 2015). Staphylococcal cassette chromosome *mec* (SCC*mec*) element subtypes were confirmed using the web-based SCC*mec*Finder tool (https://cge.cbs.dtu.dk/services/SCCmecFinder/) (Kaya *et al.,* 2018).

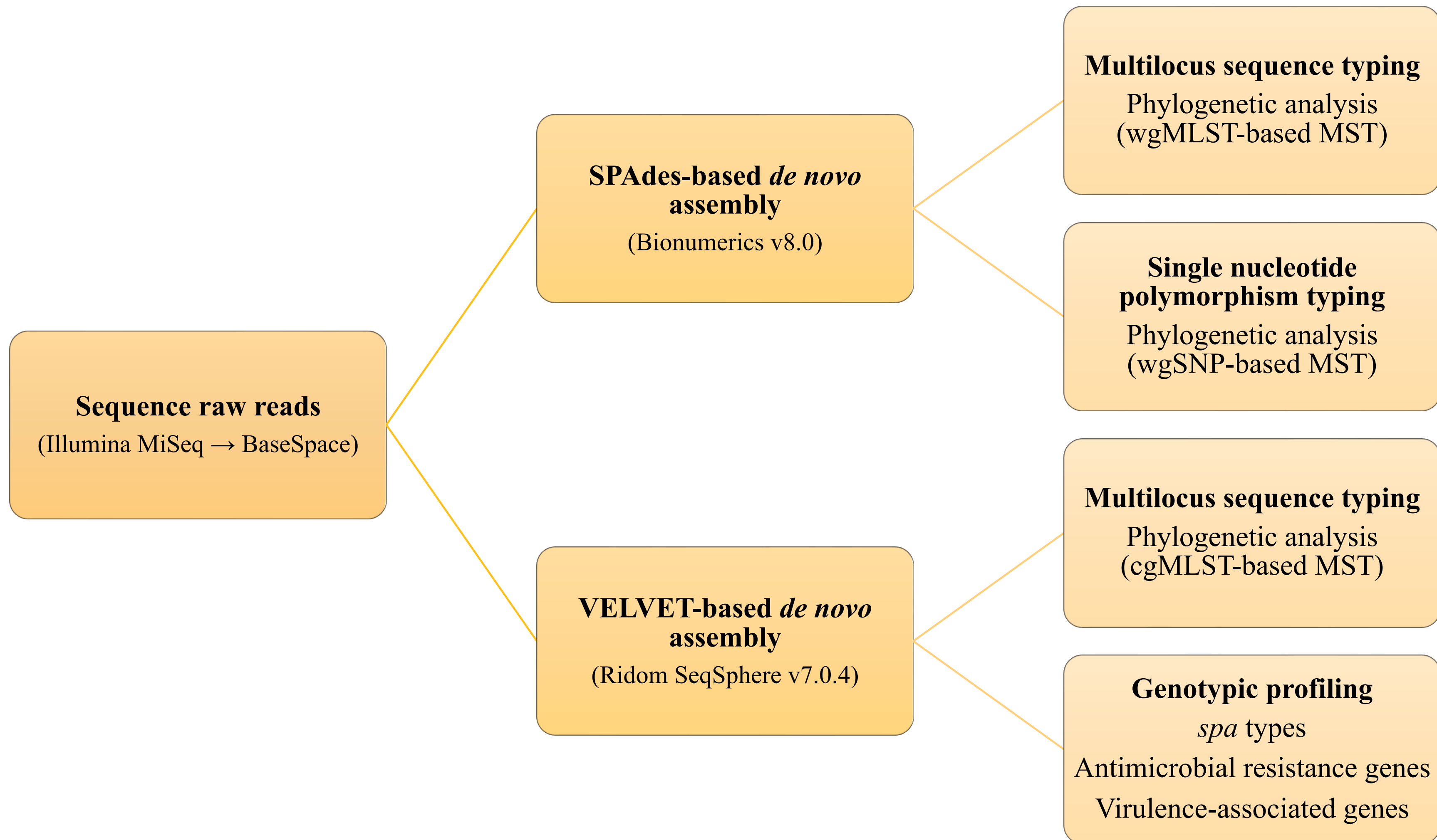


Figure 2.2. Simplified workflow of the whole genome data processing and analysis tools employed in the present study. Abbreviations: wg, whole-genome; cg, core-genome; MLST, multilocus sequence typing; MST, minimum spanning tree.

2.6.3 Multi-locus sequence typing

To accurately distinguish isolates from one another and determine relatedness, both traditional and novel multi-locus sequence typing approaches were employed using BioNumerics v8.0 (Applied Maths) and SeqSphere+ v7.0.4 (Ridom) (Fig. 2.2).

2.6.3.1 Core-genome multi-locus sequence typing

Core-genome MLST (cgMLST) was performed using SeqSphere+ v7.0.4 (Ridom) software with the cgMLST scheme previously described by Leopold *et al.,* 2014. The scheme targets 1,861 conserved genes within the core-genome loci, including the seven traditional MLST housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*). Following *de novo* assembly using SeqSphere's VELVET pipeline v1.2.10 (Ridom), the query isolates underwent the Basic Local Alignment Search Tool (BLAST)-based pairwise comparison against 40 publicly available *S. aureus* reference genomes using the pre-defined SeqSphere+ task templates (Ridom) (Altschul *et al.,* 1997).

2.6.3.2 Whole-genome multi-locus sequence typing

Whole-genome MLST (wgMLST) typing was performed using BioNumerics v8.0 (Applied Maths) software with two different automated pipelines. The *S. aureus* wgMLST scheme within BioNumerics v8.0 (Applied Maths) software was designed to analyse 3,904 gene loci, thus providing higher typing resolution. This included the 1,861 core set of genes previously defined by Leopold *et al.* 2014, alongside *S. aureus* accessory loci. Firstly, the incorporated SPAdes assembly software v3.7.1 within BioNumerics v8.0 (Applied Maths) performed assembly-free allele calling on the sequence reads directly, to determine which loci were present/absent and confirm MLST profiles. The software used a *k*-mer approach (pairwise comparison of a set of genomes based on all the possible subsequences/substrings of length '*k*' in the sequence data), with a default *k*-mer length of 35 bases applied to all sequence reads. Only loci with a minimum total coverage of 3× (minimum forward and reverse coverage of 1×) were retained for wgMLST profiling, as per BioNumerics v8.0 (Applied Maths) recommendations. Secondly, a BLAST search was carried out on *de novo* assembled genomes of the study isolates against the alleles of the seven classical MLST housekeeping genes and 31 publicly available reference genome sequences. This approach is known as assembly-based calling, and a default minimum similarity

threshold (minimum BLAST similarity between one of the reference genomes and the study isolate) of 80% was applied.

2.6.4 Single nucleotide polymorphism analysis

For further strain typing and confirmation of genetic relatedness between isolates, a whole genome single nucleotide polymorphism (wgSNP) analysis approach was employed using Bionumerics v8.0 (Applied Maths) (Fig. 2.2). For this analysis, a suitable reference genome closely related to the query genomes was used to identify SNP distribution in the isolates under investigation. The workflow involves choosing an appropriate reference sequence, mapping the raw sequence reads against this reference and filtering out relevant SNPs. The strict SNP filtering template on the Bionumerics v8.0 (Applied Maths) software retained only SNP positions with a minimum total coverage of 5× (minimum forward and reverse coverage of 1×). Positions with at least one unreliable (N) base, one gap or one ambiguous base (non-ACGT) were all removed, as were non-discriminatory positions between query sequences.

2.6.5 Phylogenetic tree generation

2.6.5.1 Minimum spanning trees

Minimum spanning trees (MSTs) were generated using BioNumerics v8.0 (Applied Maths) and SeqSphere+ v7.0.4 (Ridom) to visualise the identified cg/wgMLST-based allelic differences and wgSNPs between study isolates (Fig. 2.2). Bootstrap resampling (1000 replicates) using distance matrices was used to determine the similarities between isolates. The MST algorithm computed the shortest distance between isolates and visually represented this using connected nodes. The previously proposed threshold of ≤24 allelic differences or ≤15 SNPs for assigning *S. aureus* isolate relatedness (Schürch *et al.,* 2018) and inferring the likelihood of a transmission event were applied to all MSTs. Using these recommended thresholds based on previous studies (Earls *et al.,* 2018; Lepuschitz *et al.,* 2018; Schürch *et al.,* 2018), all isolates which exhibited allelic differences of 0–24 or 0–15 SNPs from one another were identified as a cluster group of closely related isolates and a grey partitioning was applied to each cluster for easier visualisation.

Chapter 3

CA-MRSA in Ireland: Investigating emerging lineages and distinct outbreaks of PVL-positive CA-MRSA in hospital settings using whole-genome sequencing

3.1. Introduction

The establishment of CA-MRSA into healthcare settings in recent years has marked a significant change in the epidemiology of MRSA (Byers and Decker, 2008; David and Daum, 2010; Sowash and Uhlemann, 2014). Historically, CA-MRSA were primarily associated with superficial SSTIs in young healthy individuals with no known MRSA risk factors (Sowash and Uhlemann, 2014). Nowadays however, CA-MRSA is frequently reported as a major cause of nosocomial MRSA infections worldwide (Byers and Decker, 2008; Sowash and Uhlemann, 2014). As CA-MRSA clones continue to gain traction within healthcare environments, distinctions between community and healthcare associations continue to blur, raising questions in relation to which clones will predominate and spread widely (Byers and Decker, 2008; David and Daum, 2010).

There is growing evidence indicating that the increased presence of CA-MRSA clones in healthcare environments eventually leads to displacement of predominant HA-MRSA clones within these settings (Cohen *et al.,* 2021; David *et al.,* 2014; Sowash and Uhlemann, 2014). CA-MRSA lineages have a separate evolutionary trajectory independent to that of HA-MRSA clones, and often demonstrate greater fitness, enhanced virulence and transmission potential, in comparison to the classic hospital clones being replaced (Otto, 2012; Watkins *et al.,* 2012). Contrary to initial classification of CA-MRSA lineages as PVL-encoding strains carrying smaller SCC*mec* elements (IV and V) and fewer resistance determinants than HA-MRSA, numerous PVL-positive and PVL-negative MDR CA-MRSA clones have emerged as nosocomial pathogens over the last two decades (Table 1.1) (Earls *et al.,* 2019; Kim *et al.,* 2007; Lepuschitz *et al.,* 2018).

In the USA for example, the PVL/ACME-positive CC8/ST8-MRSA-IV USA300 clone led to displacement of the predominant hospital CC5/ST5-MRSA-II USA100 clone, and is now a major lineage circulating worldwide (Broderick *et al.,* 2021). USA300 originally emerged in the late 1990s as a CA-MRSA clone, with initial reports indicating transmission and SSTI outbreaks amongst those in close contact within prisons, the military and sports teams (Planet, 2017). By the mid-2000s, the clone had spread into hospital emergency departments and taken hold in many healthcare facilities, rapidly becoming a leading cause of nosocomial BSIs (Nimmo, 2012; Planet, 2017). Successful introduction of USA300 into the healthcare environment necessitated the redefinition of this highly prevalent clone. USA300 is now classified as both a CA- and HA-MRSA

pathogen which frequently causes severe clinical infections while also surviving asymptomatically as part of the normal microflora of healthy individuals (Alam *et al.,* 2015). Like many other CA-MRSA strains, USA300 initially exhibited a very limited antimicrobial resistance profile and was primarily only resistant to methicillin. Nowadays however, resistance to non-β-lactams, including macrolides and fluoroquinolones is widely reported amongst USA300 isolates, along with carriage of many other plasmid-mediated resistance determinants (Nimmo, 2012; Tenover and Goering, 2009).

Similarly, replacement of predominant HA-MRSA in healthcare settings by a CA-MRSA clone has also been documented in the Indian subcontinent with the emergence of the 'Bengal Bay' clone (Blomfeldt *et al.,* 2017; Monecke *et al.,* 2013a). This PVL-positive CC1/ST772-MRSA-V clone was first isolated as part of a community setting study in India in 2004, before quickly overtaking the previously dominant HA ST239-MRSA-III clone in the healthcare environment (Broderick *et al.,* 2021; Steinig *et al.,* 2019). Like USA300, the ST772 Bengal Bay clone is also primarily associated with SSTIs, but more severe clinical manifestations including necrotizing pneumonia and BSIs have been observed (Steinig *et al.,* 2019). Transmission of Bengal Bay MRSA has now extended beyond the Indian subcontinent, with recent and frequent reports of the clone in the Middle East, Africa, Australia and Europe, including in Ireland (NMRSARL, 2021; Senok *et al.,* 2020; Steinig *et al.,* 2019). Brennan *et al.,* 2012, reported localised outbreaks of the ST772 Bengal Bay clone in two separate neonatal intensive care units (NICUs) in Ireland between 2010 and 2011. A HCW was identified as the probable index case based on recent travel and hospitalisation during this period. Subsequent investigations into its widespread dissemination suggested travel history to the Indian subcontinent and familial associations with this region were primary contributing factors (Blomfeldt *et al.,* 2017; Steinig *et al.,* 2019). Recent studies on ST772 Bengal Bay MRSA isolates also indicate that the lineage has evolved to acquire the MDR profiles of classic HA-MRSA clones, carrying a more extensive arsenal of antimicrobial resistance genes, compared to other CA-MRSA strains (Blomfeldt *et al.,* 2017; Monecke *et al.,* 2013a). Monecke *et al.,* 2013a, recently described the presence of staphylococcal enterotoxin A (*sea*) and the PVL cytotoxin genes, *lukS/F-PV* on the same prophage within archetypal ST772-MRSA-V Bengal Bay isolates. This is a unique marker for the Bengal Bay clone as the *sea* gene is typically associated with β-haemolysin gene (*hlb*) converting phages rather than PVL-

encoding phages (Wamel *et al.,* 2006). Additionally, the single dominant clade (ST772-A) of the Bengal Bay clone that has successfully spread globally acquired a chromosomally-integrated MDR plasmid encoding the antimicrobial resistance genes *blaZ, aphA3, msrA, mphC and bcrAB* in the early 1990s, alongside a PVL-encoding bacteriophage, small SCC*mec* V element and numerous other virulence factors (Steinig *et al.,* 2021; Steinig *et al.,* 2019).

In Europe, the prevalent CA-MRSA population is diverse and numerous strain types are currently circulating and competing within both hospital and community settings (Junie *et al.,* 2018; Romero and de Souza da Cunha, 2021). Frequent importation of CA-MRSA clones, including USA300 and Bengal Bay ST772 is often reported, and although these clones are dominant epidemic lineages rapidly spreading within the USA and India respectively, neither have established the same dominance in Europe (Turner *et al.,* 2019). In recent years, numerous outbreaks have been linked to the diverse CA-MRSA strains circulating widely across Irish hospitals, including the PVL-positive Bengal Bay clone and the introduction of a novel PVL-negative ST78-MRSA-IVa (NMRSARL, 2021). Earls *et al.*, 2019, also recently described the emergence and circulation of a PVL-negative European CC1/ST1-MRSA-IV in Ireland. This MDR CA-MRSA clone is thought to have originated from South-Eastern Europe, before spreading across Europe and becoming endemic in Germany, Romania and numerous Irish hospitals (Earls *et al.,* 2021).

Currently, ST22-MRSA-IV (also known as EMRSA-15) is one of the clinically relevant HA-MRSA clones circulating in Europe. In Ireland particularly, this endemic PVL-negative clone is the predominant cause of MRSA nosocomial infections and accounts for the majority of MRSA BSIs (70–80%) (NMRSARL, 2017; Broderick *et al.,* 2021). Although in recent years, a noticeable decrease in the number of MRSA BSIs caused by ST22-MRSA-IV has occurred. As the prevalence of PVL-negative ST22-MRSA-IV decreases, the proportion of PVL-positive MRSA isolates being submitted to the NMRSARL for investigation has gradually increased (Fig. 3.1) (Shore *et al.,* 2014). This includes PVL-positive ST22-MRSA which differ from the nosocomial EMRSA-15 clone. The NMRSARL reported detection of the *pvl* genes in 16.8% of all non-BSI MRSA isolates submitted for investigation in 2022, a significant increase from 8.8% in 2011 and 0.2% in 2002 (NMRSARL, 2021; Shore *et al.,* 2014). These PVL-positive

isolates typically harbour SCC*mec* types IV or V and predominantly belong to CC5, CC8 or CC30 (Rossney *et al.,* 2007; Shore *et al.,* 2014). The rise in PVL-positive MRSA in Ireland has been attributed to an increase in outbreaks and clusters observed in healthcare settings in recent years (NMRSARL, 2021).

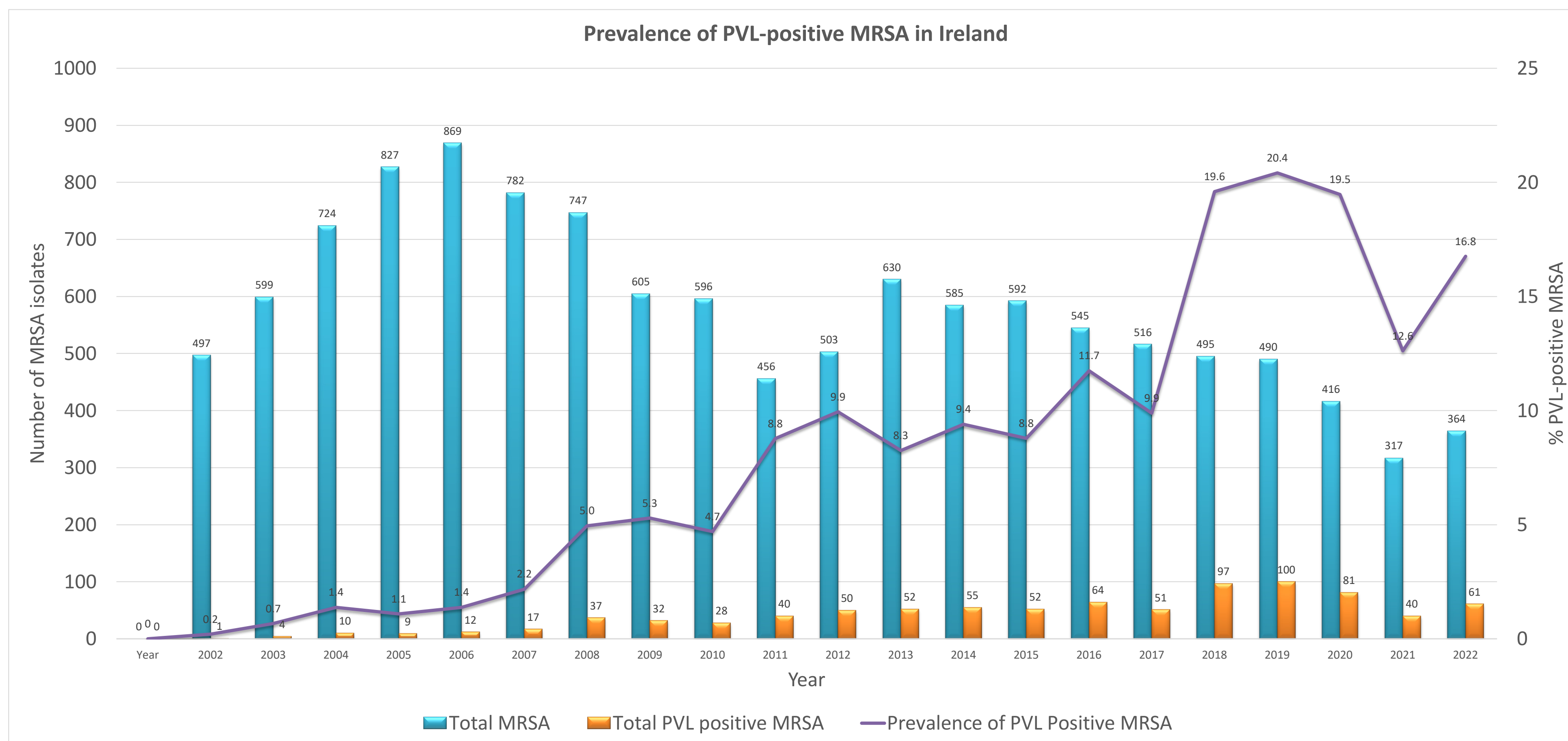


Figure 3.1. Increasing prevalence of PVL-positive MRSA in Ireland between 2002 and 2022. Total numbers of MRSA and PVL-positive MRSA submitted to the National Reference Laboratory have been reported previously for the years 2002-2011 (Shore *et al*., 2014) and from 2013-2022 (NMRSARL Annual Reports).

For the purpose of reducing the substantial burden of MRSA on public health and IPC efforts, it is important to monitor the complex dynamics and characteristics of the constantly evolving MRSA population within the wider community in Ireland. To date, a very limited number of wide-scale studies and reports are available on the CA-MRSA population in Ireland (Broderick *et al.,* 2021). Furthermore, there are currently no WGS-based surveillance systems in place investigating the prevalence and introduction of novel CA-MRSA clones into the healthcare environment from the ever-growing reservoirs of MRSA in the community. WGS provides the highest resolution for strain discrimination through use of cgMLST schemes for broad scale population structure investigations and epidemiological analyses or wgMLST schemes for tracking transmission and investigating infection outbreaks (Bernaquez *et al.,* 2021). WGS-based surveillance techniques greatly outweigh conventional approaches, allowing for better characterisation of transmission routes, detection and monitoring of outbreaks and also identification of emerging lineages. The development of high-throughput WGS over the last decade has transformed MRSA research and provides a new gold standard for highly informative molecular and epidemiological investigation of microbial pathogens.

The purpose of the present study was to use WGS to investigate infection outbreaks associated with PVL-positive CA-MRSA strains occurring in Irish hospitals in recent years and to examine the epidemiology and population structure of CA-MRSA in Ireland in order to identify the likely origins of these outbreaks. To further highlight the occurrence of localised CA-MRSA outbreaks in Irish hospital settings, these outbreak isolates were compared within the context of similar international strains. The population structure analysis in this study focused primarily on CA-MRSA associated with patients routinely attending maternity hospitals in Ireland, as three outbreaks investigated between 2011 and 2020 occurred in maternity, neonatal or paediatric units. Isolates recovered from maternity hospital patients provide an accurate representation of the general CA-MRSA population in Ireland, as patients attending maternity services are typically healthy individuals with minimal MRSA risk factors, including limited exposure to the healthcare environment and antibiotics (Holm *et al.,* 2021). Broderick *et al.*, 2021, recently reported on a three-year study in an Irish maternity hospital which showed that the prevalence of MRSA colonisation in pregnant patients in Ireland was approximately 1.6%. Another study carried out in the USA in 2009 which involved screening of pregnant patients and patients in labour also revealed that 2.1% of these

patients carried MRSA (Reusch *et al.,* 2008). These findings are in agreement with data reported by the Centers for Disease Control and Prevention (CDC) on the overall prevalence of MRSA carriage in the general population, which indicated that about two in every 100 individuals are asymptomatic carriers of MRSA (CDC, 2019). Additionally, MRSA is increasingly being associated with outbreaks in NICUs in Ireland and internationally (Alsubaie *et al.,* 2012; Brennan *et al.,* 2012; Madigan *et al.,* 2018), with horizontal transmission from colonised parents to neonates being considered a major route of entry and spread of MRSA within this environment (Holm *et al.,* 2021).

3.2 Materials and Methods

3.2.1 Isolate collection

A total of 372 MRSA isolates recovered between 1998–2022 were investigated in the current study. The collection comprised of outbreak-associated isolates, Irish and international comparator reference isolates (CRFs), a collection of CA-MRSA isolates representative of circulating CA-MRSA populations in Ireland and ST22-specific CRFs, as ST22 is the predominant lineage circulating in Ireland. Isolates were stored as described in Chapter 2, Section 2.2.1. A breakdown of the isolate collection is provided below.

3.2.1.1 Outbreak isolates

Forty-six MRSA isolates from 36 individuals suspected of being involved in several distinct infection outbreaks between 2011 and 2020 (Table 3.1) were submitted to the NMRSARL for routine analyses. These outbreak-associated isolates were recovered in three separate Irish hospitals (H1–H3) from patients (N=35) and a HCW (N=1) who had previously attended or worked at one of these hospitals. Eleven of these isolates were recovered from members of two separate family clusters (FCs), each of which had at least one member who had attended the emergency department or who was hospitalised in H2 during 2011, 2012, or 2018 due to an MRSA infection (Table 3.1).

3.2.1.2 Irish comparator reference isolates for outbreak investigation

Twenty MRSA isolates also submitted to the NMRSARL were included as CRFs due to their similarity to the outbreak-associated isolates. These isolates included PVL-positive MRSA identified as *spa* types t002, t008, and t127 recovered between 2014 and 2019 from community general practice clinics, regional Irish hospitals, or Dublin-based teaching hospitals other than those included in the present study (Tables 3.2 and 3.3).

Table 3.1. Forty-six MRSA outbreak-associated isolates investigated (2011–2020)

CC[a]	No. of isolates	No. of isolates investigated [*N*] Hospital [*N*]	Sources [*N*]	Recovery dates	*spa* types[b]	PVL(+/-)
CC5	15	H3	Maternity unit [10], Special care baby unit [3], Paediatric emergency department [1], Outpatient department [1]	2018–2020	t002	PVL+ [14] PVL- [1]
	4	H3	Special care baby unit [2], Paediatric unit(1), Staff member [1]	2019	t4667	PVL+
CC8	15	H1 [9] H2 [6]	Cardiac unit [6], Neonatal intensive care unit [5], Outpatient department [2], Gynaecology [1], Paediatric outpatient department [1]	2017–2018	t008	PVL+
	1	H1	Emergency department	2018	t723	PVL+
CC1	8	H2	Family cluster 1: Carriage [2], Superficial infection [2] Family cluster 2: Carriage [2], Superficial infection [1], Invasive infection [1]	2011–2018	t127	PVL+
CC97	3	H2	Family cluster 2: Carriage [3]	2018	t267	PVL-

[a] CC groups assigned based on hybridisation patterns obtained from DNA microarray profiling (Abbott, Jena, Germany)

[b] *spa* types assigned using Ridom Seqsphere+ v4.1 (Ridom GmbH, Münster, Germany)

Abbreviations: CC, Clonal Complex; H, Hospital; *spa*, staphylococcal protein A type; PVL, Panton-Valentine Leukocidin

Table 3.2. Twenty-seven comparator reference isolates included in the outbreak study (2014-2019)

CC[a]	No. of isolates	No. of isolates investigated [*N*] Hospital [*N*]	Sources [*N*]	Recovery dates	*spa* types[b]	PVL(+/-)	References/Biosample no.
Irish CRFs							
CC5	4	H4 [1], H11 [1], H12 [1], H13 [1]	General medicine [2], Emergency department [1], Paediatric unit [1]	2018–2019	t002	PVL+	This study
CC8	10	H2 [1], H3 [1], H5 [1], H6 [1], H8 [1], H10 [1], H12 [2], GP-Dublin [2]	Emergency department [2], General practice surgery Dublin [2], Emergency department - regional hospital [1], Intensive care unit Dublin teaching hospital [1], Regional hospital [1], Hospital [1), Dialysis [1], Not known [1]	2018–2019	t008	PVL+	This study
CC1	4	H7 [1], H12 [1], GP-Regional [2]	General practice submitted through a regional hospital [2], Not known [2]	2014–2018	t127	PVL+	This study
CC149	1	H11	Outpatient department	2018	t002	PVL+	This study
CC852	1	H11	General medicine	2018	t127	PVL+	This study
International CRFs							
CC5	2	International	United Arab Emirates-human [1], Saudi Arabia-human [1]	NK	t002	PVL+	SAMN15214112 SAMN15214113/ Monecke *et al.,* 2013b
CC1	2	International	United Arab Emirates-human [1], Egypt-bovine [1]	NK	t127	PVL+	SAMN15214126 SAMN15214127
CC97	3	International	United Arab Emirates-human [1], Saudi Arabia-human [1], Germany imported from Egypt-poultry meat [1]	2014 [1] NK [2]	t267 [2] NK [1]	PVL-	SAMN15214128/ Müller *et al.,* 2016 SAMN15214129/ Monecke *et al.,* 2012 SAMN15214130

[a] CC groups assigned based on hybridisation patterns obtained from DNA microarray profiling (Abbott, Jena, Germany) for the Irish isolates.

[b] *spa* types assigned using Ridom Seqsphere+ v4.1 (Ridom GmbH, Münster, Germany) for the Irish isolates.

Abbreviations: CC, Clonal Complex; H, Hospital; GP, general practitioner; *spa*, staphylococcal protein A type; PVL, Panton-Valentine Leukocidin; NK, not known.

Table 3.3. Molecular characterisation, antimicrobial resistance and virulence gene profiles of 46 CA-MRSA outbreak isolates and 27 CRFs from the present study

	No. of isolates investigated [*N*]						
Source[a]	**Individuals [*N*]**	**Isolates [*N*]**	***spa*-ST-SCC*mec* [*N*]**	**Antibiotic resistance**	**AR genes**	**PVL(+/-)**	**IEC type**
CC8-MRSA							
H1	8	10	t008-ST8-IVa [9] t723-ST8-IVa [1]	Ap, Kn, Nm, Er, Cp [2] Ap, Cp [8]	*aphA3*, *blaZ, fosB, lmrP, mecA, mph*(C), *mprF, msr*(A) [2] *blaZ, fosB, lmrP, mecA, mprF* [8]	PVL+ [10]	B [8] C [1] E [1]
H2	2	6	t008-ST8-IVa [6]	Ap, Cp [6]	*blaZ, fosB, lmrP, mecA, mprF* [6]	PVL+ [6]	B [6]
CRFs	10	10	t008-ST8-IVa [10]	Kn, Nm, Ap, Er, Cp [5] Kn, Nm, Ap, Er, Cp, Mp [1] Ap, Cp [1] Ap, Er [1] Ap, Er, Cp [2]	*aphA3*, *blaZ, fosB, lmrP, mecA, mph*(C), *mprF [*2] *aphA3*, *blaZ, fosB, lmrP, mecA, mph*(C), *mprF, msr*(A) [3] *aphA3*, *blaZ, fosB, lmrP, mecA, mph*(C), *mprF, msr*(A), qacC [1] *blaZ, fosB, lmrP, mecA, mph*(C), *mprF* [1] *blaZ, fosB, lmrP, mecA, mph*(C), *mprF, msr*(A) [3]	PVL+ [10]	B [9] E [1]
CC5-MRSA							
H3	18	19	t002-ST5-IVc [15] t4667-ST88-V [4]	Ap [14] Ap, Cp [1] Gn, Kn, Tb, Ap, Cp [4]	*blaZ, fosB, lmrP, mecA, mprF* [15] *aac-aphD, blaZ, mecA, mprF* [4]	PVL+ [18] PVL- [1]	B [2] G [16] F [1]
CRFs	7	7	t002-ST5-IVc [6] t002-ST149-IVc [1]	ND [2] Ap, Er [3] Ap [1]	*blaZ, fosB, lmrP, mecA, mprF* [3] *blaZ, erm*(C), *fosB, lmrP, mecA, mprF* [3] *blaZ, fosB, fusc, lmrP, mecA, mprF* [1]	PVL+ [7]	G [7]

Table 3.3 continued overleaf

Source[a]	Individuals [*N*]	Isolates [*N*]	*spa*-ST-SCC*mec* [*N*]	Antibiotic resistance	AR genes	PVL(+/-)	IEC type
CC1, CC97-MRSA							
H2-FC1	3	4	t127-ST1-V+*fus*+*tir*+*ccr*A1 [4]	Gn, Kn, Nm, Tb, Ap, Fd, Te [4]	*aacA-aphD, aphA3, blaZ, fusC, mecA, mprF* [4]	PVL+ [4]	D [4]
H2-FC2	5	7	t127-ST1-V+*fus*+*tir*+*ccr*A1 [4] t267-ST97-V+*fus* [3]	Gn, Kn, Tb, Ap, Er, Fd [4] Gn, Kn, Tb, Ap, Fd [3]	*aacA-aphD, erm*(C), *fusC, mecA, mprF* [4] *aacA-aphD, blaZ, fusC, lmrP, mecA, mprF* [3]	PVL+ [4] PVL- [3]	D [4] E [3]
CRFs	10	10	t127-ST1-V(*fus*+*tir*+*ccr*A1) [4] t267-ST97-V+*fus [*3] t127-ST1-IV(*fus*+*tir*+*ccr*A1) [1] t127-ST859-V [1] t127-ST1-IV [1]	Gn, Kn, Nm, Tb, Ap, Fd, Te [1] Ap, Er, Fd [1] Gn, Ak, Kn, Nm, Tb, Fd [1] Ap, Te, Tp [1] Kn, Nm, Tb, Ap, Te, Tp [1] ND [5]	*aacA-aphD, blaZ, lmrP, mecA, mprF* [2] *aacA-aphD, fusC, lmrP, mecA, mprF* [1] *aacA-aphD, aphA3, blaZ, erm*(C), *fusC, mecA, mprF* [1] *aacA-aphD, aphA3, blaZ, fusC, lmrP, mecA, mprF* [1] *aacA-aphD, blaZ, lmrP, mecA, mprF, tet*(K) [1] *erm*(C), *fusC, lmrP, mecA, mprF* [1] *aacA-aphD, blaZ, fusC, lmrP, mecA* [1] *blaZ, lmrP, mecA, mprF, tet*(K) [1] *aadD, blaZ, lmrP lnuA, mecA, mprF, tet*(K) [1]	PVL+ [7] PVL- [3]	D [1] E [3] G [6]

[a]Isolates recovered from patients with admission histories to hospitals H1–H3. Isolates associated with H3 recovered from patients following discharge.

Abbreviations: *spa*, staphylococcal protein A type; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mec*; AR, antimicrobial resistance; PVL, Panton-Valentine leukocidin; ND, not determined; +, positive; -, negative; H, hospital; FC, family cluster; CRFs, comparator reference isolates. Ap, ampicillin; Gn, gentamicin; Ak, amikacin; Kn, kanamycin; Nm, neomycin; Tb, tobramycin; Er, erythromycin; Fd, fusidic acid; Te, tetracycline; Cp, ciprofloxacin; Tp, trimethoprim.

3.2.1.3 International comparator reference isolates for outbreak investigation

Seven international CRFs were selected following comparison of the DNA microarray profile patterns (see Section 3.2.4) of isolates recovered from H3 and each H2-associated family cluster (FC) to an international *S. aureus* DNA microarray profile database comprising >25,000 *S. aureus* isolates from humans and animal sources. The seven CRFs selected exhibited highly similar array patterns to those of the FC isolates and consisted of two PVL-positive ST5 MRSA from humans, two PVL-positive ST1 MRSA from human (*N*=1) and bovine (*N*=1) hosts, and three PVL-negative ST97 MRSA from humans (*N*=2) and poultry meat (*N*=1) in three countries in the Middle East (Egypt, Saudi Arabia and United Arab Emirates [UAE]) (Table 3.2).

3.2.1.4 Comparative investigation of the population structure of outbreak- and non-outbreak-associated CA-MRSA

A total of 330 Irish MRSA isolates recovered from infected patients and screening samples between 2011–2022 and submitted to the NMRSARL were investigated as part of the CA-MRSA population structure study (Table 3.4). This included the 46 outbreak isolates described above in Section 3.2.1.1 and the 20 Irish CRFs described in Section 3.2.1.2. Isolates were recovered from patients in 13 different Irish hospitals (H1–H13) (*N*=326), a Dublin-based General Practitioner (GP) (*N*=2) and a regional GP (*N*=2) (Table 3.4). The majority of these isolates represented CA-MRSA isolates recovered from maternity units in H1 (*N*=212), H2 (*N*=25) and H3 (*N*=46). The remaining isolates (*N*=47) were similar isolates recovered from a children's hospital H4 (*N*=10) and various teaching hospitals (H5[2], H6[6], H7[2], H8[3], H9[8], H10[4], H11[3], H12[4], H13[1]) and GPs (*N*=4) across Ireland.

Table 3.4. Epidemiological and clinical information for 330 CA-MRSA Irish isolates investigated

ST	No. of isolates	No. of isolates investigated(*N*) Hospital [*N*][a]	*spa* types [*N*]	Recovery dates	PVL(+/-)	SCC*mec* type [*N*]	Antibiotic resistance [*N*]
ST1	42	H1 [27], H2 [9], H3 [2], H4 [1], H7 [1], GP-regional [2]	t127 [38], t1784 [1], t2279 [1], t5388 [2]	2011–2022	PVL+ [12], PVL- [30]	IVa [25] IVc [2] V [15]	Ap [41], Gn [15], Ak [1], Kn [36], Nm [31], Sp [20], Tb [16], Er [30], Fd [16], Te [24], Rf [1], Ln [4], Cp [11], Tp [3]
ST5	75	H1 [44], H2 [1], H3 [22], H4 [2], H6 [2], H9 [1], H12 [2], H13 [1]	t002 [41], t053 [2], t062 [2], t10467 [1], t111 [1], t127 [1], t1400 [1], t1802 [3], t2383 [1], t242 [1], t311 [16], t319 [2], t442 [1], t509 [1], t688 [1]	2014–2022	PVL+ [18], PVL- [57]	II [2] IVa [7] IVc [30] IVg [8] IVh [3] V [22] NT [2]	Ap [74], Gn [3], Ak [3], Kn [5], Nm [5], Sp [1], Tb [3], Er [25], Fd [23], Te [4], Rf [1], Ln [23], Cp [38], Cl [1], Tp [24]
ST6	18	H1 [16], H5 [1], H9 [1]	t304 [16], t648 [1], t711 [1]	2014–2022	PVL- [18]	IVa [16] IVc [1] V [1]	Ap [16], Gn [2], Kn [3], Nm [1], Tb [3], Er [8], Te [4], Ln [8], Cp [6], Tp [2]
ST7	1	H1	t289	2021	PVL-	NT	Ap
ST8	48	H1 [29], H2 [9], H3 [2], H5 [1], H6 [1], H8 [1], H10 [1], H12 [2], GP-Dublin [2]	t008 [35], t064 [2], t1476 [5], t20561 [1], t334 [1], t4146 [1], t451 [1], t723 [1], ND [1]	2016–2022	PVL+ [33], PVL- [15]	IVa [34] IVc [1] IVd [2] V [4] NT [7]	Ap [48], Gn [5], Kn [20], Nm [14], Tb [5], Er [22], Fd [5], Te [5], Ln [6], Cp [42], Cl [1], Tp [9]
ST22	61	H1 [48], H3 [1], H4 [2], H6 [1], H7 [1], H8 [2], H9 [4], H10 [1], H11 [1]	t032 [23], t002 [1], t005 [3], t020 [2], t022 [5], t025 [1], t10279 [1], t13735 [2], t14500 [1], t1612 [2], t17846 [1], t19070 [1], t19933 [1], t223 [2], t2231 [1], t2892 [1], t2933 [1], t2945 [3], t309 [2], t3841 [1], t4422 [1], t4623 [1], t515 [1], t608 [1], t852 [2]	2014–2022	PVL+ [7], PVL- [54]	IVa [6] IVc [5] IVh [49] V [1]	Ap [61], Gn [6], Ak [1], Kn [6], Nm [2], Sp [2], Tb [5], Er [21], Fd [24], Te [3], Rf [2], Ln [11], Cp [51], Tp [11]

Table 3.4 continued overleaf

ST	No. of isolates	Hospital [*N*][a]	*spa* types [*N*]	Recovery dates	PVL(+/-)	SCC*mec* type [*N*]	Antibiotic resistance [*N*]
ST30	11	H1 [10], H6 [1]	t012 [2], t019 [5], t021 [1], t304 [1], t779 [1], ND [1]	2014–2022	PVL+ [8], PVL- [3]	IV [1] IVc [9] NT [1]	Ap [11], Kn [1], Nm [1], Tb [1], Er [1], Fd [4], Cp [2], Tp [1]
ST39	1	H1	t007	2014	PVL-	II	Ap, Gn, Kn, Nm, Tb, Er, Ln
ST45	6	H1 [4], H4 [10, H9 [1]	t132 [2], t362 [2], t266 [1], t20358 [1]	2014–2022	PVL- [6]	IV [1] IVb [4] NT [1]	Ap [6], Er [5], Fd [1], Cp [1]
ST59	3	H1 [3]	t976 [1], t437 [1], t3952 [1]	2017–2021	PVL+ [1], PVL- [2]	IVa [1] V [1] NT [1]	Ap [3], Kn [1], Nm [1], Sp [1], Er [2], Ln [1]
ST72	1	H1	t324	2022	PLV-	IVc	Ap [1], Cp [1]
ST80	1	H1	t934	2022	PVL-	IVa	Ap [1], Er [1], Te [1], Cp [1]
ST88	10	H1 [4], H3 [4], H4 [2]	t4667 [4], t5562 [2], t690 [2], t786 [1], t13249 [1]	2018–2022	PVL+ [7], PVL- [3]	IVa [6] V [4]	Ap [10], Gn [4], Kn [5], Nm [1], Tb [4], Er [2], Fd [1], Te [1], Cp [6], Cl [1], Tp [1]
ST97	6	H2 [3], H6 [1], H10 [2]	t267 [4], t12805 [2]	2016–2019	PVL- [6]	V [4] NT [2]	Ap [6], Gn [4], Kn [4], Tb [4], Fd [4]
ST130	1	H3	t843	2021	PVL-	NT	Ap
ST149	2	H9 [1], H11 [1]	t002 [2]	2016–2018	PVL+ [1], PVL- [1]	IVa [2]	Ap [2], Er [1], Fd [2], Cp [1]
ST152	1	H1	t355	2022	PVL+	IVa	Ap, Kn, Nm, Tb, Er, Te, Cp, Tp
ST508	2	H1 [2]	t19352 [1], t20681 [1]	2020–2022	PVL- [2]	IVc	Ap [2], Cp [1]
ST580	1	H1	t1039	2021	PVL-	IVa	Ap
ST672	9	H1 [5], H2 [1], H3 [3]	t3841 [8], t3175 [1]	2020–2022	PVL- [9]	V [9]	Ap [9], Gn [1], Kn [2], Nm [2], Tb [1], Er [3], Fd [8], Cp [9], Tp [8]

Table 3.4 continued overleaf

ST	No. of isolates	Hospital [*N*][a]	*spa* types [*N*]	Recovery dates	PVL(+/-)	SCC*mec* type [*N*]	Antibiotic resistance [*N*]
ST772	12	H1 [1], H3 [11]	t657 [12]	2017–2021	PVL+ [1], PVL- [11]	V [11] IVc [1]	Ap [12], Gn [12], Ak [1], Kn [12], Nm [12], Tb [12], Er [12], Fd [1], Cp [12], Tp [12]
ST779	4	H1 [4]	t878 [4]	2015–2020	PVL- [4]	V [4]	Ap [4], Fd [4]
ST789	4	H1 [2], H2 [2]	t091 [3], t1943 [1]	2018–2022	PVL- [4]	V [4]	Ap [4], Gn [4], Ak [1], Kn [4], Nm [4], Tb [4], Te [3], Cp [4], Tp [3]
ST852	1	H11	t127	2018	PVL+	III	Ap, Te, Tp
ST1649	1	H1	t701	2022	PVL-	IVc	Ap, Cp, Tp
ST2149	1	H1	t008	2022	PVL-	IVc	Ap, Cp
ST2689	1	H1	t5608	2022	PVL-	IVa	Ap, Kn, Nm, Er, Fd, Te, Tp
ST2884	1	H1	t693	2022	PVL+	IVa	Ap, Er, Cp, Tp
ST3738	1	H4	t032	2021	PVL-	IVh	Ap, Fd, Cp
ST5050	2	H1 [2]	t321 [1], t5977 [1]	2021–2022	PVL- [2]	IVa [2]	Ap [2], Kn [2], Nm [2], Sp [2], Er [2], Te [2], Cp [2]
ST5084	1	H1	t359	2019	PVL-	IVc	Ap
ST6953	1	H4	t1476	2021	PVL-	NT	Ap, Gn, Kn, Tb, Cp, Tp

[a] Isolates recovered from 13 different hospitals and two General Practitioners between 2011–2022.

Abbreviations: N, number; H, hospital; GP, General Practitioner; ND, not determined – *spa* types could not be determined using *in-silico* techniques on the available genomic sequence data; ST, sequence type; PVL, Panton-Valentine leukocidin; +, positive; -, negative; Ap, ampicillin; Gn, gentamicin; Ak, amikacin; Kn, kanamycin; Nm, neomycin; Sp, streptomycin; Tb, tobramycin; Er, erythromycin; Fd, fusidic acid; Te, tetracycline; Rf, rifampicin; Ln, lincomycin; Cp, ciprofloxacin; Cl, chloramphenicol; Tp, trimethoprim.

3.2.1.5 International comparator reference isolates for ST22-MRSA-IV investigation

Of the 330 CA-MRSA isolates recovered from Irish hospitals, 61 were identified as ST22-MRSA of which 7/61 were PVL-positive. The current study sought to compare these Irish PVL-positive ST22-MRSA isolates with similar international strains. For this comparative investigation, all available 30,142 *S. aureus* assembled genomes in the pubMLST database (https://pubmlst.org) (accessed 28th March 2023) were screened to identify all ST22 *S. aureus* strains. A total of 5,149 ST22 genomes were identified as 'exact/nearest matches' to the ST22 loci combinations. Using *lukS/F-PV* PVL PCR primers (PVL-F; ATGTCTGGACATGATCCAA and PVL-R; AACTATCTCTGCCATATGGT) designed by Ma *et al*., 2006, an in-silico search was performed on these 5,149 ST22 genomes to identify PVL-positive strains. A total of 417 genomes were positive for the PVL PCR product. Only a subset of these international strains (*N*=34) had epidemiological information available regarding country and year of isolation (Table 3.5). One of these strains was excluded from the analysis due to the poor quality of the WGS files available.

An extensive search of the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA)/GenBank, European Nucleotide Archive (ENA) databases and publicly available literature using the following search parameters: 'ST22, CC22, PVL-positive, *Staphylococcus aureus*' provided four additional international isolates. Two of these isolates were excluded from the analysis due to the poor quality of the WGS files available. The sequences of the other two isolates that were deposited into the ENA database by a research laboratory from the University of Nottingham (UK) were included in this study (Table 3.5). In total, genomic data for 35 international CRF strains (21 MRSA and 14 MSSA strains) recovered in eight different countries (Australia, China, Germany, India, Namibia, Nepal, UK and USA) were included as epidemiologically unrelated PVL-positive ST22 comparators for the PVL-positive ST22 Irish CA-MRSA isolates in this study (Table 3.5).

3.2.2 NMRSARL molecular characterisation

All Irish isolates originally submitted to the NMRSARL underwent species identification and phenotypic antimicrobial susceptibility testing as described in Chapter 2, Section 2.3. The NMRSARL also performed *spa* typing on these 330 isolates as described in Chapter 2, Section 2.3.3. Ninety-four different *spa* types were identified for 328/330 (99.4%)

isolates and the two remaining isolates were non-typeable (Table 3.1). The predominant *spa* types detected were t002 (12.5%), t127 (11.9%), t008 (11%), t032 (7.9%), t304 (5.2%), t311 (4.9%) and t657 (3.7%) (Table 3.6).

Table 3.5. Seven PVL-positive ST22-MRSA isolates from Ireland and 35 international CRFs investigated

Country[a]	Isolates [*N*]	Recovery dates	*spa*-ST-SCC*mec* [*N*]	AR genes [*N*]	Virulence factor genes	IEC type [*N*]	References/ pubMLST ID/ Biosample no.
(a) NMRSARL isolates							
Ireland	7	2017–2022	t005-ST22-IVc [1] t005-ST22-IVa [1] t309-ST22-ND [2] t852-ST22-IVc [2] t4422-ST22-IVc [1]	*blaZ* [7], *ccrA2/B2* [6], *ccrAA* [1], *mecA* [7], *mecR-truncated* [5], *erm*(C) [3], *lmrP* [7], *aac-aphD* [6], *aadD* [3], *dfrA* [5], *ugpQ* [7], *mprF* [7], *sdrM* [7], *tet*(K) [1]	*aur* [7], *ebpS* [7], *eno* [7], *hl* [7], *hla* [7], *hlgA/B/C* [4], *hlgB/C* [3], *lukF/S-PV* [7], *lukX/Y* [7], *seg* [7], *sei* [7], *sec* [2], *sel* [2], *sspA/B/P* [7], *tst1* [2]	B [7]	This study
(b) pubMLST search isolates							
Australia	2	Unknown	t1802-ST22-IVc [1] t005-ST22-IVa [1]	*blaZ* [2], *ccrA2/B2* [2], *mecA* [2], *mecR-truncated* [2], *lmrP* [2], *aac-aphD* [2], *aadD* [1], *dfrA* [2], *ugpQ* [2], *mprF* [2], *sdrM* [2]	*aur* [2], *ebpS* [2], *eno* [2], *hl* [2], *hla* [2], *hlgA/B/C* [2], *lukF/S-PV* [2], *lukX/Y* [2], *seg* [2], *sei* [2], *sspA/B/P* [1], *sspA/P* [1], *tst1* [1]	B [2]	36217 37770
China	5	2019	t309-ST22-MSSA [4] t309-ST22-ND [1]	*blaZ* [5], *ccrAA/C* [1], *mecA* [1], *lmrP* [5], *mprF* [5], *ugpQ* [1], *sdrM* [5], *erm*(C) [1]	*aur* [5], *ebpS* [5], *eno* [5], *hl* [5], *hla* [5], *hlgA/B/C* [5], *lukF/S-PV* [5], *lukX/Y* [5], *seg* [5], *sei* [5], *sspA/B/P* [5]	B [5]	45763 45766 45767 45769 45770
Germany	5	2002–2007	t1977-ST22-IV [2] t845-ST22-MSSA [1] ND-ST22-IV [2]	*blaZ* [5], *ccrA2/B2* [4], *mecA* [4], *mecR*-truncated [4], *mprF* [5], *qacC* [1], *fusC* [4], *lmrP* [5], *ugpQ* [4], *sdrM* [5]	*aur* [5], *ebpS* [5], *eno* [5], *hl* [5], *hla* [5], *hlgA/B/C* [5], *lukF/S-PV* [5], *lukX/Y* [5], *seg* [5], *sei* [5], *sspA/B/P* [4], *sspB/P* [1]	B [5]	6927 7087 7210 7570 7767
India	1	2017	t1328-ST22-IVa	*blaZ, ccrA2/B2, mecA, mecR-*truncated, *lmrP, aac-aphD, dfrA, mecA, tet* [K], *ugpQ, mprF, sdrM*	*aur, ebpS, eno, hl, hla, hlgA/B/C, lukF/S-PV, lukX/Y, seg, sei, sspA/B/P*	B	43059

Table 3.5 continued overleaf

Country[a]	Isolates [*N*]	Recovery dates	*spa*-ST-SCC*mec* [*N*]	AR genes [*N*]	Virulence factor genes	IEC type [*N*]	References/ pubMLST ID/ Biosample no.
Namibia	2	2009	ND-ST22-MSSA [2]	*blaZ* [2], *lmrP* [2], *aac-aphD* [2], *dfrA* [1], *mprF* [2], *sdrM* [2]	*aur* [2], *ebpS* [2], *eno* [2], *hl* [2], *hla* [2], *hlgA/B* [2], *lukF/S-PV* [2], *lukX/Y* [2], *seg* [2], *sei* [2], *sspA/B/P* [2]	B [2]	7459 7529
Nepal	3 (animal samples)	2016	t005-ST22-IVa [2] t309-ST22-IVa [1]	*ccrA2/B2* [3], *mecA* [3], *mecR*-truncated [3], *lmrP* [3], *aac-aphD* [2], *dfrA* [3], *erm*(C) [2], *ugpQ* [3], *mprF* [2], *sdrM* [3]	*aur* [3], *ebpS* [3], *eno* [3], *hl* [3], *hla* [3], *hlgA/B/C* [2], *hlgA/B* [1], *lukF/S-PV* [3], *lukX/Y* [3], *seg* [2], *sei* [1], *sspA/B/P* [3], *tst1* [3]	B [3]	42631 42633 42634
UK	12	1998–2011	t474-ST22-IVc [2] t474-ST22-IVd [1] t005-ST22-IVc [2] t852-ST22-IVc [1] t1304-ST22-MSSA [1] t1372-ST22-MSSA [1] t1977-ST22-IV [1] t13828-ST22-MSSA [1] ND-ST22-IVa [1] ND-ST22-IVc [1]	*blaZ* [12], *ccrA2/B2* [9], *mecA* [9], *mecR*-truncated [9], *lmrP* [12], *aac-aphD* [6], *aadD* [6], *dfrA* [5], *erm*(C) [4], *fusC* [1], *ugpQ* [9], *mprF* [12], *sdrM* [12]	*aur* [12], *ebpS* [12], *eno* [12], *hl* [12], *hla* [12], *hlgA/B/C* [9], *hlgB/C* [1], *hlgB* [1], *hlgA/B* [1], *lukF/S-PV* [12], *lukX/Y* [12], *seg* [12], *sei* [12], *sec* [2], *sspA/B/P* [11], *sspB/P* [1]	B [9] *Hlb*-intact [2] C [1]	6805 6875 6876 7665 7680 8602 8733 11170 11429 11622 11889 13558
USA	3	2009–2012	t005-ST22-MSSA [2] ND-ST22-IVc [1]	*blaZ* [3], *ccrA2/B2* [1], *mecA* [1], *mecR*-truncated [1], *aac-aphD* [3], *lmrP* [3], *aadD* [1], *dfrA* [3], *ugpQ* [1], *mprF* [3], *sdrM* [3]	*aur [3], ebpS [3], eno [3], hl [3], hla [3], hlgA/B/C [3], lukF/S-PV [3], lukX/Y [3], seg [3], sei [3], sem [2], sen [3], seo [3], seu [3], sspA/B/P [2], sspB/P [1]*	B [2] C [1]	27002 39268 39328
(c) NCBI search isolates							
UK	2	2009	t005-ST22-MSSA [2]	*blaZ* [2], *lmrP* [2], *aac-aphD* [2], *dfrA* [2], *mprF* [2], *sdrM* [2]	*aur* [2], *ebpS* [2], *eno* [2], *hl* [2], *hla* [2], *hlgA/B/C* [2], *lukF/S-PV* [2], *lukX/Y* [2], *seg* [2], *sei* [2], *sspA/B/P* [2]	B [2]	SAMEA5605434 SAMEA5605435

[a] Irish NMRSARL isolates and international isolates recovered from pubMLST and extensive literature search.

Abbreviations: ND, not determined –*spa* type could not be determined using in-silico techniques on the available genomic sequence data; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; PVL, Panton-Valentine leukocidin; +, positive; -, negative; IEC, immune evasion cluster, ID, identification; no., number.

Table 3.6. *spa* type and repeat profiles of 330 CA-MRSA isolates recovered from Ireland investigated in the present study

spa type [*N*]	*spa* type repeat succession	*spa* type [*N*]	*spa* type repeat succession
t002 [44]	26-23-17-34-17-20-17-12-17-16	t19352 [1]	08-16-02-23-16-34-34-17-34-16-34
t005 [3]	26-23-13-23-31-05-17-25-17-25-16-28	t1943 [1]	07-23-21-17-34-12-23
t007 [1]	15-12-16-16-16-16-02-25-17	t19933 [1]	26-23-23-13-23-31-340-17-31-29-17-24-25-17-25-16-28
t008 [36]	11-19-12-21-17-34-24-34-22-25	t20358 [1]	09-02-16-34-34-34-17-34-16-16-34
t012 [2]	15-12-16-02-16-02-25-17-24-24	t20561 [1]	11-19-12-02-17-34-24-34-34-22-25
t019 [5]	08-16-02-16-02-25-17-24	t20681 [1]	08-16-02-23-16-34-17-34-16-34
t020 [2]	26-23-31-29-17-31-29-17-25-17-25-16-28	t2892 [1]	26-23-13-23-31-29-17-31-29-17-25-16-28
t021 [1]	15-12-16-02-16-02-25-17-24	t223 [2]	26-23-13-23-05-17-25-17-25-16-28
t022 [5]	26-23-13-23-31-29-17-31-29-17-25-17-25-16-28	t2231 [1]	26-23-31-29-17-31-29-17-25-16-16-28
t025 [1]	26-23-23-13-23-29-17-31-29-17-25-17-25-16-28	t2279 [1]	07-23-21-16
t032 [24]	26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28	t2383 [1]	08-16
t053 [2]	26-23-17-34-17-20-17-12-17-34	t242 [1]	26-23-17-13-17-20-17-12-17-16
t062 [2]	26-23-17-12-17-16	t266 [1]	09-02-16-13-13-13-17-34-16-34
t064 [2]	11-19-12-05-17-34-24-34-22-25	t267 [4]	07-23-12-21-17-34-34-34-33-34
t091 [3]	07-23-21-17-34-12-23-02-12-23	t289 [1]	26-23-21-17-34-12-23-02-12-23
t10279 [1]	26-23-23-13-23-31-31-17-31-29-17-25-17-25-16-28	t2933 [1]	26-23-13-23-05-17-25-17-25-16-16-28
t1039 [1]	08-16-02-25-02-25-24-65-17-25	t2945 [3]	26-23-23-13-23-31-29-23-31-29-17-25-17-25-16-28
t10467 [1]	26-23-17-34-17-20-17-66-16	t304 [17]	11-10-21-17-34-24-34-22-25
t111 [1]	26-23-17-16	t309 [2]	26-23-05-17-25-17-25-16-28
t127 [40]	07-23-21-16-34-33-13	t311 [16]	26-23-17-34-20-17-12-17-16
t12805 [2]	07-23-12-21-17-34-34-34-33-34-34-34-34-33-34	t3175 [1]	26-22-17-20-17-12-17-16
t132 [2]	09-34-16-34	t319 [2]	26-23-17-34-20-17-12-16
t13249 [1]	07-21-34	t321 [1]	07-23-16-34-33-13
t13735 [2]	26-23-23-13-23-23-13-23-31-29-17-31-29-17-25-16-16-28	t324 [1]	07-23-12-12-17-20-17-12-12-17
t1400 [1]	26-23-17-34-20-17-12-17	t334 [1]	11-12-21-17-34-22-25
t14500 [1]	26-23-23-13-23-31-29-17-31-29-17-25-17-25-25-17-25-25-16-28	t355 [1]	07-56-12-17-16-16-33-31-57-12

Table 3.6 continued overleaf

spa type [*N*]	*spa* type repeat succession	*spa* type [*N*]	*spa* type repeat succession
t1476 [6]	11-10-17-34-24-34-22-25	t359 [1]	07-23-12-21-17-34-34-33-34
t1612 [2]	26-23-23-13-23-31-29	t362 [2]	09-34
t1784 [1]	07-34-33-13	t3841 [9]	26-22-17-20-17-12-17-16-16
t17846 [1]	26-23-23-34-23-31-29-17-31-29-17-25-17-25-28	t3952 [1]	04-20-17-31-16-34-34
t1802 [3]	26-16-16-28	t4146 [1]	11-12-12-34-22-25
t19070 [1]	26-23-23-13-23-31-29-23-31-29-17-25-16-28	t437 [1]	04-20-17-20-17-25-34
t4422 [1]	07-23-13-23-31-05-17-25-17-24-25-16-28	t442 [1]	35-17-34-17-20-17-12-17-16
t451 [1]	11-12-05-17-34-24-34-22-25	t693 [1]	07
t4623 [1]	26-23-13-23-31-29-132-17-31-29-17-25-17-25-16-28	t701 [1]	11-10-21-17-34-24-34-22-25-25
t4667 [4]	07-13-13-13-34-33-13	t711 [1]	04-21-17-34-24-34-22-25
t509 [1]	26-23-17-20-17-12-17-16	t723 [1]	11-19-12-34-22-25
t515 [1]	26-23-23-13-23-31-29-17-31-29-17-25-16-16-28	t779 [1]	08
t5388 [2]	07-23-21-16-34-33-21-13	t786 [1]	07-12-21-17-13-34-34-33-34
t5562 [2]	07-12-21-17-13-34-33-34	t843 [1]	04-82-17-25-17-25-25-16-17
t5608 [1]	26-23-17-34-17-13-17-20-17-12-17-16	t852 [2]	07-23-13-23-31-05-17-25-17-25-16-28
t5977 [1]	07-23-16	t878 [4]	26-23-17-34-21-25-33-16
t608 [1]	26-23-31-29-17-25-17-25-16-28	t934 [1]	07-23-12-34-34-34-34-33-34
t648 [1]	11-21-17-34-24-34-22-25	t976 [1]	04-20-17-20-31-16-34
t657 [12]	26-23-13-21-17-34-33-34	ND [2]	
t688 [1]	26-23-17-34-17-16		
t690 [2]	07-12-21-17-13-13-34-34-34-33-34		

Abbreviations: *spa*, staphylococcal protein A; N., number; ND, not determined.

3.2.3 Genomic DNA isolation

All Irish isolates underwent genomic DNA extraction as described in Chapter 2, Section 2.4.1.

3.2.4 DNA microarray profiling

DNA microarray profiling was performed to preliminarily identify isolates deemed similar to the outbreak-associated isolates based on *spa* types and carriage of *pvl* genes. Array profiling can infer STs, SCC*mec* types and details virulence factors and antimicrobial resistance profiles prior to WGS-based confirmation. Genomic DNA extraction was performed as described in Section 2.4.1. DNA microarray analysis was performed using the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH]) as per manufacturer's instructions. The kit consisted of all required reagents and microtitre strips containing DNA microarray chips. Each chip consisted of 333 probes corresponding to ~170 *S. aureus* target sequences (including allelic variants of the genes), including species-specific antimicrobial resistance genes, virulence factor genes, SCC*mec* genes and other pathogenicity markers. The DNA microarray process detects the presence or absence of these genes. The protocol is detailed below.

3.2.4.1 Linear PCR amplification and biotin labelling

Each genomic DNA sample was diluted to the desired concentration (0.5–1.5 µg/µl) using sterile molecular-grade water to a final volume of 5 µl. This was combined with a master-mix containing 4.9 µl of B1 labelling buffer and 0.1 µl of B2 DNA polymerase. The 10 µl sample underwent PCR amplification and biotin labelling using a PCR thermal cycler (Biometra TOne) at the following conditions: 96°C for 5 min, 45 cycles of 62°C for 20 s, 72°C for 40 s and 96°C for 1 min.

3.2.4.2 Array hybridisation

Each 10 µl amplified PCR product was mixed with 90 µl of C1 hybridisation buffer. The microtitre wells within the array strip were washed with 200 µl of sterile water, the water was discarded and 100 µl of C1 buffer was added to each well. The microarray strip was then incubated at 55°C for 2 min at 550 rpm in a BioShake iQ thermoshaker (Q instruments, Jena, Germany). Following incubation, the C1 buffer was discarded and the PCR samples with the added buffer was distributed into the microarray strip (one sample per well). The strip was incubated at 55°C for 1 h at 550 rpm in a thermoshaker to allow

for hybridisation to occur. Following hybridisation, each well of the microarray strip was washed three times with 200 µl of C2 wash buffer.

3.2.4.3 Streptavidin-horseradish-peroxidase (HRP)-conjugation and staining

For conjugation, a 1:100 ratio of the conjugation mixture (1 µl of C3 HRP-reagent buffer and 99 µl of C4 conjugate buffer) was prepared for each sample. This 100 µl C3/C4 mix was added to each well of the microarray strip, followed by a 10 min incubation at 30°C and 550 rpm to allow for binding of the streptavidin-HRP with the biotin-labelled target of each sample. After conjugation, each well was washed twice with 200 µl of C5 wash buffer. Following this, staining of the bound HRP-conjugate was performed by addition of 100 µl of reagent D1 which contained the tetramethylbenzidine (TMB) HRP-substrate to each well. The strip was incubated at room temperature for 5 min without agitation of the strip. The D1 reagent was then fully discarded from each well and the strip was analysed immediately.

3.2.4.4 Data acquisition and analysis

Analysis of the microarray strip was performed using the ArrayMate Reader software (Abbott [Alere Technologies GmbH]) as per manufacturer's instructions. The software scans for signals of positive hybridisation events and generates digital images of the microarray. The generated image depicts either weak or strong signals which indicates the quality of the samples analysed. The arraymate readout interprets detection of each specific target sequence/marker as either 'positive', 'negative' or 'ambigiuous' based on a previously described algorithm (Monecke *et al.,* 2008). The microarray results of each sample were then compared to the array profiles of previously characterised strains stored within the ArrayMate (Abbott [Alere Technologies GmbH]) public database in order to perform assignment of STs, CCs and SCC*mec* types.

3.2.5 Whole-genome sequencing

All Irish study isolates underwent short-read WGS as described in Chapter 2, Section 2.5.1.

3.2.6 Bioinformatic analyses

All study isolates underwent *de novo* assembly, *spa* type confirmation, SCC*mec* subtyping, MLST, *in silico* antimicrobial resistance profiling and virulence gene identification as described in Chapter 2, Section 2.6.

3.2.6.1 wgSNP analysis

With an appropriately chosen reference sequence, wgSNP offers the ultimate resolution in determining relatedness between epidemiologically linked isolates (Uelze *et al.,* 2020). To ensure SNPs are adequately detected, a reference strain which is closely-related to the set of isolates under investigation is required (Uelze *et al.,* 2020). The WGS sequence of this reference strain must also be a high quality closed reference genome with a low number of contigs in order for it to permit calling of SNP positions with high accuracy (Uelze *et al.,* 2020). For this investigation, a total of 73 isolates (46 Irish outbreak isolates, 20 Irish CRFs and seven international CRFs) recovered from infected patients and screening samples between 2011–2020 underwent wgSNP-based analysis in Bionumerics v8.0 (Applied Maths) as described in Chapter 2, Section 2.6.4.

3.2.6.2 Minimum spanning trees

Generation of MSTs was performed as described in Chapter 2, Section 2.6.5.1. The MSTs constructed included: (i) a wgMLST-based MST and a wgSNP-based MST with 73 isolates (46 Irish outbreak isolates, 20 Irish CRFs and seven international CRFs) in the suspected CA-MRSA outbreak study, (ii) a cgMLST-based MST with all 330 Irish isolates from the present study, (iii) a cgMLST-based MST with 61 Irish ST22-MRSA isolates from the present study. A second cgMLST-based MST with seven Irish PVL-positive ST22-MRSA isolates and 35 international PVL-positive ST22 *S. aureus* CRF isolates and lastly, (iv) a cgMLST-based MST and a wgMLST-based MST with twelve Irish ST722-MRSA isolates from the present study.

3.2.7 Serial passaging

To investigate the genomic stability of MRSA isolates over extended periods of time, serial passaging of representative isolates (*N*=4) from four different lineages (CC1, CC5, CC8 and CC97) investigated in this study was carried out. Isolates were re-activated from bead storage at -80°C and single colonies were sub-cultured onto CBA plates every 24 h for a total of 10 days as described in Section 2.4. On days 2, 4, 8 and 10 of growth, a

single colony was randomly selected to undergo DNA extraction as described in Chapter 2, Section 2.4, followed by WGS as described in Chapter 2, Section 2.5.

3.3 Results

3.3.1. WGS investigation of PVL-positive outbreak-associated clones in Irish hospitals

The 46 CA-MRSA outbreak-associated isolates investigated were recovered from 36 different individuals attending three separate Irish hospitals (H1–H3) over a nine-year period (2011–2020) (Table 3.1). In addition, 27 epidemiologically unrelated but genotypically similar CRFs were included for context; 20 of these were Irish isolates submitted to the NMRSARL from Dublin-based teaching hospitals and regional clinics (2014–2019), and seven international CRF isolates were selected from the global microarray database based on the close similarity of their array profile patterns to the corresponding outbreak-associated isolate array profiles (Table 3.2).

3.3.1.1 H1- and H2-associated t008-ST8-MRSA outbreaks

To investigate possible transmission events within and between two separate Irish hospitals, 10 ACME- and PVL-positive ST8-MRSA-IVa (USA300) isolates recovered in a Dublin-based maternity hospital (H1) between 2017–2018 and six additional isolates from a separate Dublin-based hospital (H2) were investigated using WGS (Table 3.3). The H1 isolates were recovered from eight patients from the outpatient unit (*N*=3), the gynaecology department (*N*=1), the emergency department (*N*=1) and the neonatal intesive care unit (*N*=5) (Table 3.1). The H2 isolates were recovered from two separate patients during one week within the same period of the H1 outbreak and were also identified as ACME- and PVL-positive ST8-MRSA-IVa (Table 3.3). One H1 isolate was identified as *spa* type t723, whereas the remaining 15 H1 and H2 isolates were t008 (Table 3.3). Ten CRFs identified as PVL-positive t008 ST8-MRSA-IV (Table 3.2) and the USA300 type reference strain FPR3757 (GenBank Accession no. CP000255.1 for FPR3757 WGS sequence) were also investigated for comparative purposes. WgMLST-based and wgSNP-based MSTs were constructed for these H1, H2 and CRF isolates (Figs. 3.2a and 3.3a). As the FPR3757 strain was not closely related to the isolates in the present study and its WGS sequence also contained many contigs, rendering it unsuitable for a SNP analysis, it was excluded from the wgSNP-based MST.

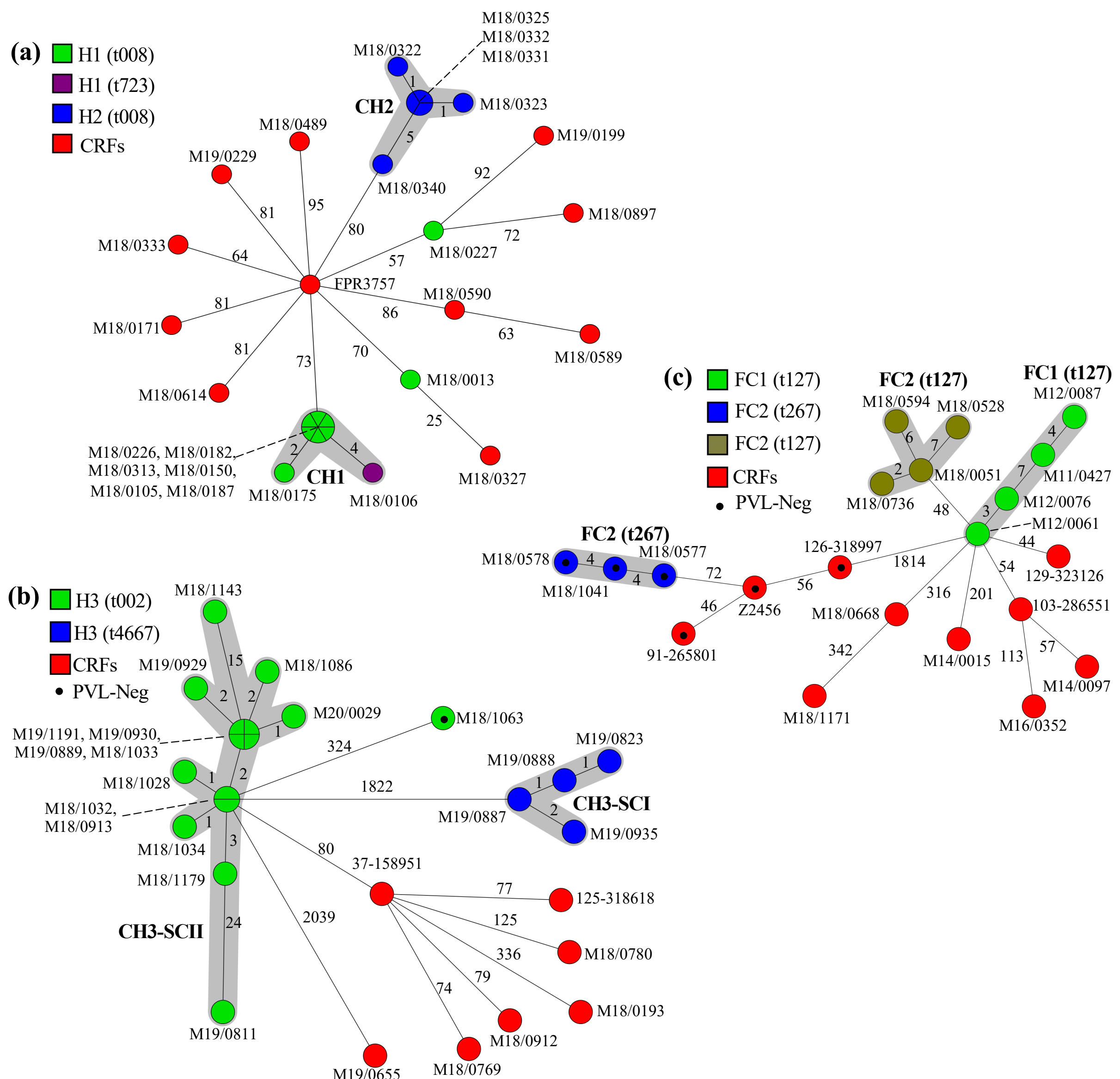


Figure 3.2. Minimum spanning trees (MSTs) based on whole-genome multi-locus sequence typing (wgMLST) analysis of the 42 Panton–Valentine leucocidin (PVL)-positive and four PVL-negative MRSA outbreak-associated isolates investigated in addition to epidemiologically unrelated but genotypically similar comparator reference isolates (CRFs). In each MST, MRSA isolates recovered from separate hospitals or families and identified as distinct *spa* types are indicated by separate colours. Genotypically similar but epidemiologically unrelated CRFs included for comparative purposes are indicated in each MST as red circles. Closely related clusters of isolates (≤24 wgMLST allelic differences [Schürch *et al*., 2018]) are outlined with grey shadowing. A black dot in the centre of each circle is used to indicate PVL-negative isolates which were included in the study as they were identified as the same *spa* types/STs as the outbreak-associated PVL-positive MRSA and were recovered

in the same hospital over the relevant time periods. The PVL-negative t267 isolates were included as they were also recovered from one of the families affected by the PVL-positive t127 MRSA lineage. The numbers on each branch indicate the numbers of wgMLST allelic differences detected between neighbouring isolates. The epidemiological information for each isolate is provided in Table 3.1 and CRF in Table 3.2. Genotypic data for outbreak isolates and CRFs is provided in Table 3.3. **(a)** wgMLST-based MST constructed from the t008, ST8-IVa MRSA isolates associated with hospitals 1 and 2 (H1 (*N*=10) and H2 (*N*=6)), CRFs (*N*=10) and the USA300 reference strain FPR3757. The two distinct clusters, CH1 and CH2, refer to isolates recovered from hospitals H1 and H2, respectively. **(b)** wgMLST-based MST constructed from the t002-ST5-IVc (*N*=15) and t4667-ST88-V (*N*=4) isolates associated with hospital 3 (H3) and CRFs (*N*=7). With the exception of the PVL-negative isolate M18/1063, all t002-ST5-IVc isolates formed a distinct subcluster (CH3–SCI), and all four t4667-ST88-V isolates formed a second subcluster (CH3–SCII). **(c)** wgMLST-based MST constructed from the t127-ST1-V+*fus*+*tirS*+*ccrA1*(*N*=8) and t267-ST97-V+*fus* (*N*=3) isolates recovered from multiple members of two distinct families (FC1 (*N*=4) and FC2 (*N*=7)), of which each family had at least one member that either attended the emergency department of, or was admitted to, hospital 2 (H2), and 10 epidemiologically unrelated CRFs. Three distinct subclusters were apparent (FC1 (t127), FC2 (t127) and FC2 (t267)), each of which consisted of all of the isolates identified as each distinct *spa* type and from each separate family.

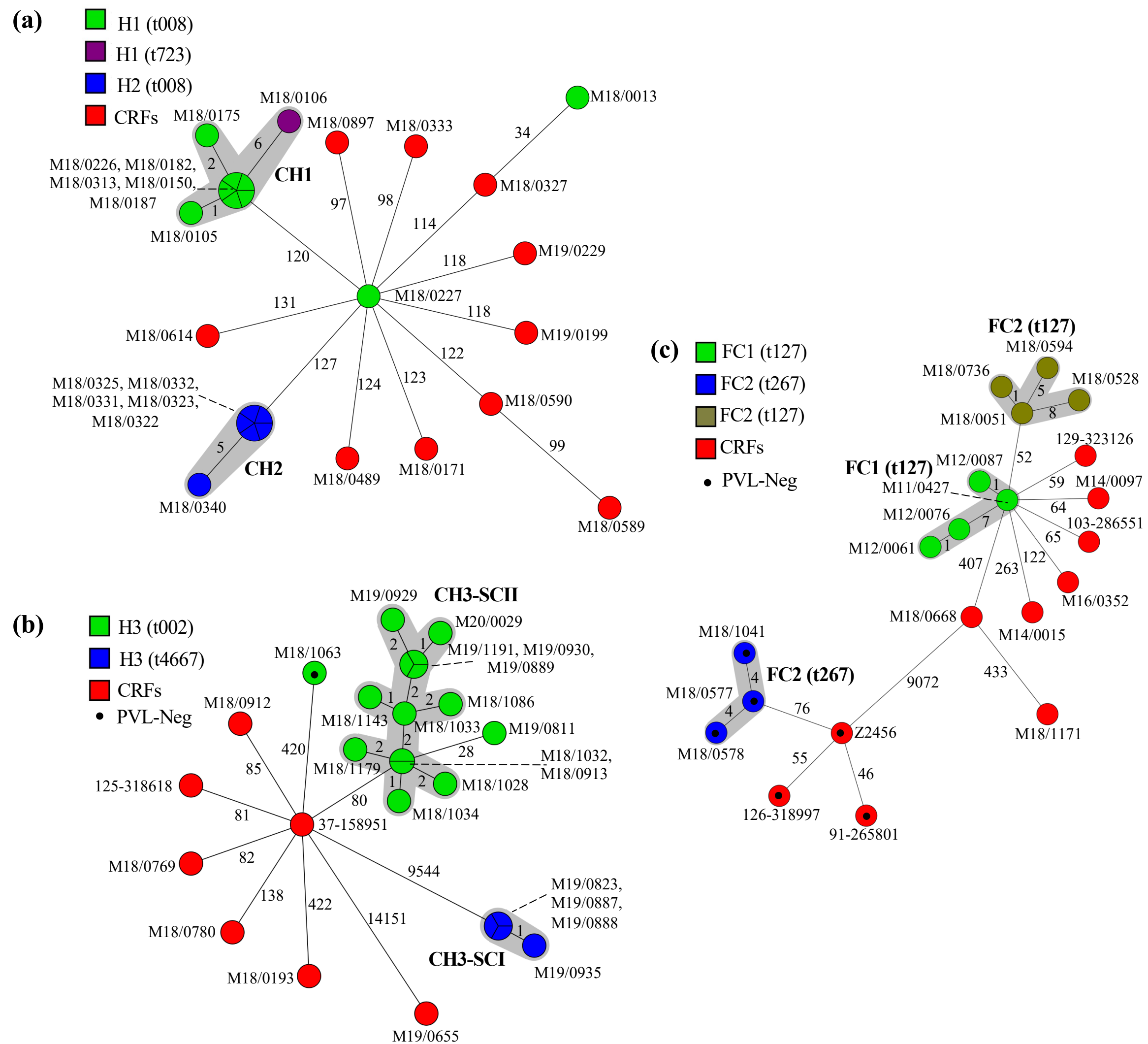


Figure 3.3. Minimum spanning trees (MSTs) based on the single nucleotide polymorphism (SNP) analysis of the 42 Panton–Valentine leucocidin (PVL)-positive and four PVL-negative MRSA outbreak-associated isolates investigated in addition to epidemiologically unrelated but genotypically similar comparator reference isolates (CRFs). In each MST, MRSA isolates recovered from distinct hospitals or families and identified as distinct *spa* types are indicated by separate coloured circles. Genotypically similar but epidemiologically unrelated CRFs included for comparative purposes are indicated in each MST as red circles. Closely related clusters of isolates (≤15 SNPs) are outlined with grey shadowing (Schürch *et al.,* 2018). A black dot in the centre of each circle is used to indicate PVL-negative isolates which were included in the study as they were identified as the same *spa* types/STs as the outbreak-associated PVL-positive MRSA and were recovered in the same hospital over the relevant time periods. The PVL-negative t267 isolates were included as they were also recovered from one of the families affected by the PVL-positive t127 MRSA lineage. The number on each branch indicates

the numbers of SNVs detected between neighbouring isolates. The epidemiological information for each isolate is provided in Table 3.1 and CRF in Table 3.2. Genotypic data for outbreak isolates and CRFs is provided in Table 3.3. **(a)** wgSNP-based MST constructed from the t008, ST8-IVa MRSA isolates associated with hospitals 1 and 2 [H1 (*N*=10) and H2 (*N*=6)] and CRFs (*N*=10). The two distinct clusters CH1 and CH2, refer to isolates recovered from hospitals H1 and H2, respectively. **(b)** wgSNP-based MST constructed from the t002-ST5-IVc (N=15) and t4667-ST88-V (*N*=4) isolates associated with hospital 3 (H3) and CRFs (*N*=7). With the exception of M18/1063 and M19/0811, all t002-ST5-IVc isolates formed a distinct sub-cluster (CH3-SCII), and all four t4667-ST88-V isolates formed a second sub-cluster (CH3-SCI). **(c)** wgSNP-based MST constructed from the t127-ST1-V+fus+tirS+ccrA1 (*N*=8) and t267-ST97-V+fus (N=4) isolates recovered from multiple members of two distinct families [FC1 (*N*= 4) and FC2 (*N*= 7)] from which each had at least one member associated with hospital 2 (H2) and 10 epidemiologically unrelated CRFs. Three distinct sub-clusters were apparent, FC1 (t127), FC2 (t127) and FC2 (t267) each of which consisted of all the isolates identified as each distinct *spa* type and from each separate family.

The wgMLST- and SNP-based MSTs revealed two discrete clusters termed CH1 (8/10 H1 isolates) and CH2 (all six H2 isolates)) within which the majority of H1 and H2 isolates clustered, respectively (Figs. 3.2a and 3.3a). Isolates within CH1 were closely related with a median of 0 (average: 1.5; range: 0–4) allelic differences and 0 (average: 1.1; range: 0–6) SNPs. The two remaining H1 isolates (M18/0227 and M18/0013) differed from CH1 isolates by ≥73 allelic differences and ≥120 SNPs (Figs. 3.2a and 3.3a). Within CH2, isolates were also closely related with a median of 0.5 (average: 0.5; range: 0–5) allelic differences and 0 (average: 0.8; range: 0–5) SNPs. These findings suggested high genetic relatedness between the isolates belonging to each cluster. By contrast, none of the epidemiologically unrelated CRFs clustered within CH1 or CH2. The 10 ST8 CRFs and the FPR3757 reference exhibited an average of 74 allelic differences between neighbouring isolates and none clustered with CH1 or CH2. These findings indicated the occurrence of two separate MRSA outbreaks in H1 and H2 that were independent of one another and involved genetically distinct MRSA strains. The H1 outbreak involved the eight CH1 isolates while the H2 outbreak involved the six CH2 isolates. The eight closely related CH1 isolates were recovered from six separate neonates (age <1) from the NICU of H1 and from two adult patients (aged 23–39) in the emergency department and outpatient department of H1. The six CH2 isolates on the other hand were recovered from patients (aged 76–78) in a cardiac unit within H2. Whereas all 16 isolates recovered from H1 and H2 exhibited ciprofloxacin and ampicillin resistance, two distinct phenotypic antibiotic resistance patterns were identified by the NMRSARL (Table 3.3). Additionally, isolates M18/0227 and M18/0013 from H1 also exhibited resistance to erythromycin, kanamycin and neomycin (Table 3.3). By contrast, the 10 CRFs exhibited five distinct antimicrobial susceptibility patterns (Table 3.3).

3.3.1.2 H3-associated ST5 and ST88-MRSA outbreaks

To investigate two separate outbreaks caused by two distinct MRSA clones occurring concurrently within the same maternity unit of a regional Irish hospital (H3), nineteen MRSA isolates recovered from 17 separate patients and one HCW associated with this hospital were investigated using WGS (Table 3.1). These isolates were recovered between 2018–2020 from patients in the maternity ward (*N*=10), the special care baby unit (SCBU) (*N*=5), the outpatient department (*N*=1), the emergency department (*N*=1), the paediatric unit (*N*=1) and from a HCW (*N*=1) (Table 3.1). Fifteen of the isolates were recovered over a 15-month period during 2018–2020 and identified as t002 ST5-MRSA-

IVc isolates. The other four isolates were recovered during one month in 2019 and identified as t4667 ST88-MRSA-V (Table 3.2). Eighteen of the isolates were PVL-positive, and one t002 isolate was PVL-negative (Table 3.3). To confirm the occurrence of two distinct MRSA outbreaks within H3 during the same time-period, these 19 isolates were subjected to a wgMLST analysis. Five Irish t002 CRFs and two international epidemiologically unrelated PVL-positive CC5-MRSA-IV CRFs (125–318618 and 37–158951) selected on the basis of having highly similar DNA array profiles to the t002 isolates were also included for comparative purposes (Table 3.2).

The MSTs constructed based on the wgMLST- and SNP-analyses of the H3-associated isolates and seven CRFs revealed two distinct subclusters consisting of either t4667 (subcluster CH3–SCI, *N*=4 isolates) or t002 (subcluster CH3–SCII, *N*=14) (*N*=13 in the wgSNP-based MST) isolates. The PVL-negative t002 ST5-MRSA-IVc isolate from H3 (M18/1063) did not cluster with any other isolates and exhibited 324 allelic differences to the CH3–SCII subcluster (Figs. 3.2b and 3.3b). Using the pre-defined thresholds for inferring close relatedness between *S. aureus* isolates (≤24 allelic differences and ≤15 SNPs) (Schürch *et al*., 2018), all four t4667 isolates within CH3–SCI were deemed to be closely related as they exhibited a median of 1 (average: 1; range: 1–2) allelic differences and 0 (average: 0.25; range: 0–1) SNPs from one another. These four isolates were recovered from three neonatal SCBU/paediatric patients (age <1) and one HCW in H3 during a one-month period in 2019. All four yielded identical phenotypic antibiotic susceptibility profiles, and exhibited resistance to ≥3 classes of clinically relevant antibiotics.

According to the wgMLST-based MST, the 14 t002 isolates within CH3-SCII were also closely related to one another, exhibiting a median of 1 (average: 3.6; range: 0–24) allelic differences for all 14 isolates (Figs. 3.2b and 3.3b). The SNP-based MST correlated with the wgMLST-based MST tree and showed a median of 1 (average: 1.15; range: 0–2) SNP for 13 isolates with the exception of isolate M19/0811, which was separated from its nearest CH3–SCII neighbour by only 28 SNPs (Figs. 3.2b and 3.3b). Four isolates within CH3-SCII (M18/1033, M19/0889, M19/0930 and M19/1191) were genetically indistinguishable with no allelic differences observed despite being recovered from four separate patients in different units within H3 at intervals of eleven, one, and two months

apart, respectively (Fig. 3.2b and 3.3b). The 14 closely related isolates within CH3-SCII were recovered from seven separate neonates (age <1), two children (aged 2) and five adult patients (aged 24–40) in the maternity unit, SCBU, outpatient department and paediatric emergency department of H3.

The PVL-negative t002 ST5-MRSA-IVc isolate M18/1063 did not cluster within either CH3-SCI or CH3-SCII and exhibited 324 allelic differences to the CH3-SCII cluster (Fig. 3.2b). None of the CRFs clustered within CH3-SCI or CH3-SCII and exhibited a minimum of 80 allelic differences and 80 SNPs to the closest relative isolate within CH3-SCII. Subclusters CH3-SCI and CH3-SCII were separated by 1822 allelic differences and ~9544 SNPs, which strongly indicated the occurence of two distinct independent MRSA outbreaks within H3 (Figs. 3.2b and 3.3b). One outbreak involved the four t4667 CH3-SCI isolates while the second involved 14 t002 CH3-SCII isolates. All 15 t002 isolates exhibited ampicillin resistance while the PVL-negative isolate M18/1063 also exhibited ciprofloxacin resistance. All four t4667 isolates exhibited resistance to ≥3 classes of antibiotics and exhibited identical resistance profiles which also included resistance to ampicillin and ciprofloxacin (Table 3.3).

3.3.1.3 H2-associated ST1- and ST97-MRSA family outbreaks

To investigate the possible transmission of a PVL-positive CC1-MRSA between two separate families associated with the same Dublin-based hospital (H2) and also confirm the transmission of a separate distinct PVL-negative CC97-MRSA within family 2 (FC2) at the same time, eleven isolates recovered from members of these two separate unrelated families were investigated using WGS (Table 3.1). At least one member from each of the two families had attended H2 as a result of an MRSA infection. Four isolates recovered from three family members belonging to family 1 (FC1) between 2011-2012 were investigated. Of these four isolates, two samples were recovered from one individual (aged 23) within the family; once during treatment for a SSTI and also subsequently as a screening sample. Another sample was recovered from a separate family member (aged 24) who presented to the emergency department of H2 with a SSTI also. The other isolate was recovered from a screening sample of a third family member (a neonate: age <1). All four isolates were identified as PVL-positive t127 ST1-SCC*fus* MRSA (Table 3.3).

In an unrelated episode, seven isolates recovered from five family members belonging to family 2 (FC2) during a seven-month period between 2017–2018 were also investigated. Of these seven, one isolate was recovered from a family member who was an inpatient in H2 as a result of an invasive MRSA infection (aged 81) and another from a family member who presented to H2 with a superficial MRSA infection (aged 46). Two more isolates were recovered as screening samples from the individual with the previous SSTI. The three remaining isolates were recovered from screening samples of three other family members (aged 3–76). Of the seven isolates, four were also identified as PVL-positive t127 ST1-SCC*fus* isolates while three were identified as PVL-negative t267 ST97-SCC*fus* isolates (Table 3.3). The phylogenetic investigation of isolates from the two separate families also included ten CRFs. Five of these were international isolates identified as either PVL-positive ST1 (*N*=2) or PVL-negative ST97 isolates (*N*=3). The other five CRFs were Irish PVL-positive ST1 isolates recovered from hospitals separate from H2 (Table 3.2). These CRF isolates were selected as suitable comparators based on the detection of the *fusC* gene and the similarities of SCC*mec* elements harboured by these isolates which were similar to those harboured by the H2-associated family cluster isolates in this investigation.

Three separate clusters (FC1-t127, FC2-t127 and FC2-t267) were observed on the wgMLST-based and wgSNP-based MSTs constructed (Figs. 3.2c and 3.3c). Within each of these distinct FC1-t127, FC2-t127 and FC2-t267 family clusters, isolates were separated from their nearest neighbour by ≤7 allelic differences or ≤8 SNPs. Isolates belonging to FC1 (t127) were separated from their nearest neighbouring isolate by a median of 4 (average: 4.6; range: 3–7) allelic differences and 1 (average: 3; range: 1–7) SNP, which indicated that these isolates are closely-related. Isolates belonging to FC2 (t127) were separated from their nearest neighbour by a median of 6 (average: 3.8; range: 2–7) allelic differences and 5 (average: 4.7; range: 1–8) SNPs which again indicated close genetic relatedness between these clustering isolates. FC2 (t127) isolates were separated by 48 allelic differences or 52 SNPs to the FC1 (t127) isolates. The three PVL-negative t267 isolates which clustered into FC2-t267 also exhibited a median of four (average: 4; range: 4–4) allelic differences and four (average: 4; range: 4–4) SNPs (Figs. 3.2c and 3.3c). In contrast, none of the comparator strains clustered within FC1-t127, FC2-t127 or FC2-t267 (Figs. 3.2c and 3.3c). The PVL-positive ST1 CRFs were closest to the FC1-t127 cluster and exhibited a minimum of 44 allelic differences (or 59 SNPs) from an

isolate within this cluster. The three PVL-negative ST97 CRFs on the other hand were closest to the FC2-t267 cluster and exhibited a minimum of 72 allelic differences (or 76 SNPs) from an isolate within this cluster (Fig. 3.2c and 3.3c).

The FC1, FC2, and CRF t127 ST1 isolates harboured genes indicative of SCC*mec* IV, V or the SCC*mec* V subtype (V+*fus*+*tirS*+*ccrA1*) previously identified in sporadic PVL-positive CC1 isolates. The FC2 and CRF t267 ST97 isolates harboured genes indicative of SCC*mec* V+*fus* previously identified in sporadic CC97 isolates. Collectively, these findings indicated transmission of a distinct t127 strain amongst members of family 1, the transmission of another distinct t127 strain amongst members of family 2 alongside the spread of a t267 strain within family 2 also. This investigation revealed that direct transmission of the t127 isolates between the two families investigated was unlikely due to the identification of 48 allelic differences between the two t127 clusters. All eleven t127 and t267 isolates exhibited resistance to ≥3 classes of antibiotics which included phenotypic resistance to ampicillin, fusidic acid, tobramycin, gentamicin and kanamycin by all isolates (Table 3.3).

3.3.1.4 Serial-passaged isolates

The wgMLST-based analysis of the colonies randomly selected from serial passage of the representative CC1, CC5, CC8 and CC97 MRSA isolates from the PVL-positive CA-MRSA investigation revealed high genetic similarities between each passage. The CC8/ST8-MRSA-IVa isolate M18/0227 passaged derivatives exhibited an allelic difference range of 0–1, the CC5/ST5-MRSA-IVc isolate M18/1033 passaged derivatives exhibited no allelic differences while the CC1/ST1-MRSA-SCC*fus* isolate M18/0051 passaged derivatives and the CC97/ST97-MRSA-SCC*fus* isolate M18/0578 passaged derivatives revealed allelic difference ranges of 1–1 and 0–3 between each passage, respectively.

3.3.2. CA-MRSA population in Ireland

In order to investigate the general population of CA-MRSA in Ireland, 330 isolates recovered from 13 different Irish hospitals (H1–H13), a Dublin-based GP and a regional GP between 2011–2022 were investigated by WGS (Table 3.4). The majority of isolates were recovered from adult patients attending outpatient departments (61%; 201/330), while the remaining were recovered from inpatient units (20%; 67/330) or from unknown

sources (19%; 62/330). Among this collection of isolates, one sample was recovered in 2011 (0.3%), three in 2012 (0.9%), 19 in 2014 (5.8%), two in 2015 (0.6%), 15 in 2016 (4.5%), 15 in 2017 (4.5%), 72 in 2018 (21.8%), 39 in 2019 (11.8%), 39 in 2020 (11.8%), 70 in 2021 (21.2%) and 55 in 2022 (16.7%).

Epidemiological information regarding specimen site was available for the majority of isolates investigated (77%; 253/330). The majority of isolates were recovered from screening samples (72%; *N*=182), specifically nasal, throat and groin. The remaining were infection site isolates (28%; *N*=71), including samples from superficial infections and blood cultures. Information regarding age of patient at time of specimen collection was also available for the majority of isolates investigated (99%; 326/330). Isolates were mainly from adult patients (aged: 18–118) (84%, *N*=273), while the remaining were recovered from children/teenagers (aged: 2–17) (4%; *N*=13) and neonates (age: <1) (12%; *N*=40). The median age for the patient demographic was 32. As previously mentioned, 91 different *spa* types were identified by the NMRSARL for 328/330 (99.4%) isolates and the dominant *spa* types were t002 (13.4%), t127 (12.2%), t008 (11%), t032 (7.3%), t304 (5.2%), t311 (4.9%) and t657 (3.7%) (Table 3.6). The assigned *spa* types were re-confirmed for all isolates using the appropriate SeqSphere+ (Ridom GmbH) task template tool (see Chapter 2, Section 2.6.6).

3.3.2.1 MLST

Multi-locus sequence types (STs) were determined for all 330 Irish isolates from the WGS data (see Section 3.2.6). A total of 32 different STs were identified. The dominant STs were ST5 (22.7%, *N*=75), ST22 (18.4%, *N*=61), ST8 (14.5%, *N*=48) and ST1 (12.7%, *N*=42), followed by ST6 (5.4%, *N*=18), ST772 (4%, *N*=12), ST30 (3%, *N*=11), ST88 (3%, *N*=10) and ST672 (2.7%, *N*=9). Six isolates each were assigned to ST45 and ST97 (2%), four isolates to ST779 and ST789 (1%), three to ST59 (1%), and lastly, two to ST149, ST508 and ST5050 (0.6%). The remaining 15 STs identified were all associated with a single isolate each (0.3%) (Table 3.4).

3.3.2.2 Carriage of mecA and pvl lukF/S-PV genes

All Irish isolates investigated harboured the *mec*A gene. Of the 330 isolates, 27.6% (*N*=91) harboured the *lukF/S-PV pvl* genes. Within the 32 different STs identified in this study, isolates harbouring *pvl* genes were only associated with twelve of these STs (Table

3.1). The predominant ST containing PVL-positive isolates was ST8 (36.2%; 33/91), followed by ST5 (19.7%; 18/91), ST1 (13.1%; 12/91), ST30 (8.8%; 8/91), ST88 (7.7%; 7/91) and ST22 (7.7%; 7/91). The other STs (ST59, ST149, ST152, ST772, ST852 and ST2884) were all associated with only a single PVL-positive isolate each (1.1%; 1/91).

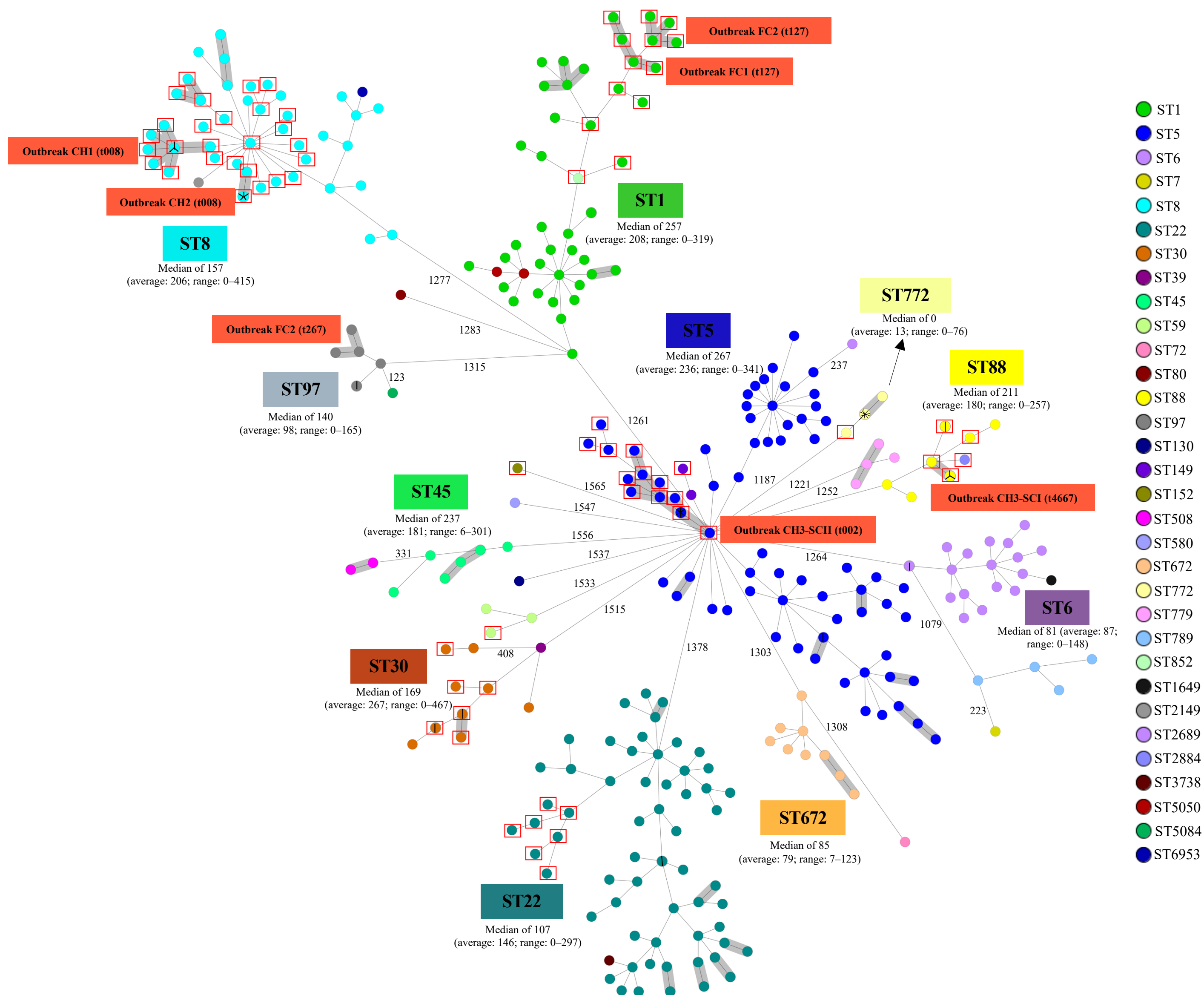
3.3.2.3 NMRSARL phenotypic antimicrobial susceptibility

The majority of isolates within the study population exhibited phenotypic resistance to the broad-spectrum β-lactam agent, ampicillin (98.8%; 326/330) (Table 3.4). A significant proportion of isolates under investigation were also phenotypically resistant to ciprofloxacin (58.8%; 194/330), erythromycin (42%; 139/330), kanamycin (31.8%; 105/330), fusidic acid (28.8%; 95/330), lincomycin (24%; 80/330), trimethoprim (24%; 80/330), neomycin (24%; 79/330), tobramycin (18.5%; 61/330), gentamicin (17.6%; 58/330) and tetracycline (15.2%; 50/330). A small number of isolates also showed resistance to streptomycin (7.9%; 26/330), amikacin (2%; 7/330), chloramphenicol (0.9%; 3/330) and rifampicin (0.9%; 3/330).

3.3.2.4 Investigation of CA-MRSA population structure using WGS

For surveillance of the CA-MRSA population circulating in Irish hospitals, a cgMLST-based MST was constructed for all 330 isolates investigated. Within the MST, all isolates were colour-coded based on ST and predominant STs as well as the ST1, ST5, ST8, ST88 and ST97 outbreaks previously investigated were also clearly labelled (Fig. 3.4). Among the collection, 91 different *spa* types and 32 distinct STs were identified. Despite this extensive genetic diversity, a significant proportion of the population belonged to four major STs, including ST5 (22.7%), ST22 (18.4%), ST8 (14.5%) and ST1 (12.7%). Analysis of the population structure revealed that isolates belonging to each of these prevalent STs were detected throughout the entire 11-year study period, whereas others were intermittently detected. ST1, ST5 and ST8 are all major strains commonly associated with CA-MRSA lineages widely circulating in Ireland. ST22 is the predominant lineage currently accounting for approximately 70–80% of all MRSA BSIs in Ireland. The majority of isolates belonging to these predominant STs were also mainly assigned to prevalent *spa* types commonly reported in MRSA isolates in Europe (Asadollahi *et al*., 2018). This included t002 amongst ST5 isolates (54.7%; 41/75), t008 amongst ST8s (72.9%; 35/48), t032 amongst ST22s (37.7%; 23/61) and t127 in ST1 isolates (90.5%; 38/42) (Table 3.4). SCC*mec* type IV and V which are typically

associated with CA-MRSA strains were also the predominant SCC*mec* elements identified within the population of isolates investigated in this study (Fig. 3.4). SCC*mec* type IV was found in 69% (228/330) of isolates with subtypes IVa (*N*=104), IVc (*N*=55) and IVh (*N*=53) being the most common. SCC*mec* type V was associated with 24% (80/330) of isolates. The remaining isolates carried SCC*mec* II (*N*=4), III (*N*=1), IVb (*N*=4), IVd (*N*=2), IVg (*N*=8), IV; subtype unknown (*N*=2) or were non-typeable (NT) strains (*N*=17).



Outbreak FC2 (t127)
Outbreak FC1 (t127)
Outbreak CH1 (t008)
Outbreak CH2 (t008)
ST8
Median of 157
(average: 206; range: 0–415)
ST1
Median of 257
(average: 208; range: 0–319)
1277
1283
Outbreak FC2 (t267)
1315
123
ST97
Median of 140
(average: 98; range: 0–165)
ST5
Median of 267
(average: 236; range: 0–341)
ST772
Median of 0
(average: 13; range: 0–76)
237
ST88
Median of 211
(average: 180; range: 0–257)
1261
Outbreak CH3-SCI (t4667)
ST45
Median of 237
(average: 181; range: 6–301)
1565
1547
1187
1221
1252
Outbreak CH3-SCII (t002)
1556
331
1537
1264
1533
1515
ST6
Median of 81 (average: 87; range: 0–148)
1079
ST30
Median of 169
(average: 267; range: 0–467)
408
1378
1303
223
1308
ST672
Median of 85
(average: 79; range: 7–123)
ST22
Median of 107
(average: 146; range: 0–297)
ST1
ST5
ST6
ST7
ST8
ST22
ST30
ST39
ST45
ST59
ST72
ST80
ST88
ST97
ST130
ST149
ST152
ST508
ST580
ST672
ST772
ST779
ST789
ST852
ST1649
ST2149
ST2689
ST2884
ST3738
ST5050
ST5084
ST6953

Figure 3.4. Minimum spanning tree (MST) based on core-genome multi-locus sequence typing (cgMLST) analysis of the 330 CA-MRSA Irish isolates (Panton–Valentine leucocidin (PVL)-positive [*N*=91] and PVL-negative [*N*=239]) investigated in the current study. In the MST, MRSA isolates were colour-coded based on their assigned sequence types (STs). Thirty-two different STs were identified in this study. The numbers on each branch indicate the number of cgMLST allelic differences detected between neighbouring STs. The median/average/range of allelic differences between isolates within each ST group is clearly labelled. Closely related clusters of isolates (≤24 cgMLST allelic differences [Schürch *et al*., 2018])are outlined with grey shadowing. A red square around a node is used to indicate a PVL-positive isolate. The predominant STs have also been clearly labelled on the MST, alongside all the outbreak-associated isolate clusters. The epidemiological information for each isolate investigated is shown in Table 3.4.

The cgMLST-based analysis revealed that isolates belonging to the same ST clustered closer together and exhibited fewer allelic differences to one another than with other neighbouring ST groups. Within STs where ≥2 isolates were present, isolates exhibited allelic differences ranging between 0 and 467. Table 3.7 provides a detailed breakdown of allelic differences based on ST grouping. In contrast, STs exhibited >1000 allelic differences to neighbouring ST groups on average (Fig. 3.4). Collectively, these findings indicate the presence of several genetically distinct variants of CA-MRSA circulating widely across Ireland.

The cgMLST phylogenetic analysis also highlighted the increase in the proportion of PVL-positive MRSA in Ireland in recent years. In particular, the clusters of PVL-positive outbreak-associated isolates stood out within the context of the overall general CA-MRSA population under investigation. These distinct outbreak isolate clusters which were primarily observed within predominant STs were either well-dispersed within the ST population (ST8, ST30 and ST88) or were localised to a subgroup branching out close to their ST groups (ST1, ST5 and ST22) (Fig. 3.4). In addition to the outbreak isolates within ST1, ST5, ST8, ST88 and ST97, this WGS investigation also revealed patterns of transmission/emergence of novel strains within some of the other circulating STs. Notably, an increase in PVL-positive ST22-MRSA, separate to the endemic PVL-negative ST22 EMRSA-15 was observed. Additionally, evidence of transmission of a PVL-negative ST772-MRSA with similarities to the PVL-positive ST772 Bengal Bay clone was also noted from the cgMLST-based MST. These STs were analysed further and the outcome of the investigation is detailed below.

Table 3.7. Allelic differences between isolates [N=330] assigned to the same sequence type in the CA-MRSA investigation

ST	No. of isolates	Allelic differences between isolates
ST1	42	Median of 257 (average: 208; range: 0–319)
ST5	75	Median of 267 (average: 236; range: 0–341)
ST6	18	Median of 81 (average: 87; range: 0–148)
ST8	48	Median of 157 (average: 206; range: 0–415)
ST22	61	Median of 107 (average: 146; range: 0–297)
ST30	11	Median of 169 (average: 267; range: 0–467)
ST45	6	Median of 237 (average: 181; range: 6–301)
ST59	3	Median of 265 (average: 270; range: 263–283)
ST88	10	Median of 211 (average: 180; range: 0–257)
ST97	6	Median of 140 (average: 98; range: 0–165)
ST149	2	115
ST508	2	18
ST672	9	Median of 85 (average: 79; range: 7–123)
ST772	12	Median of 0 (average: 13; range: 0–76)
ST779	4	Median of 49 (average: 49; range: 5–93)
ST789	4	Median of 73 (average: 72; range: 59–86)
ST5050	2	56

Abbreviation: ST, sequence type

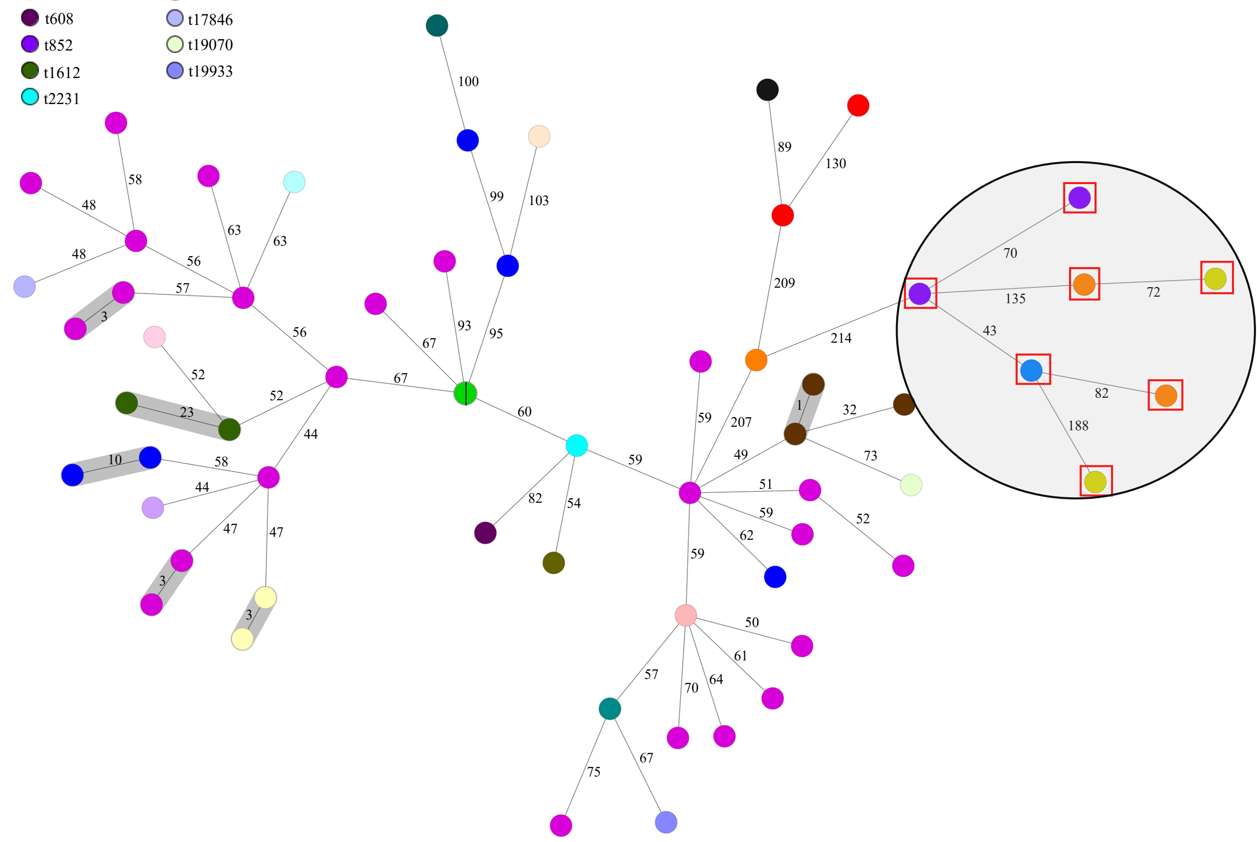
3.3.2.5 Comparative investigation of Irish PVL-positive ST22-MRSA and international PVL-positive ST22-MRSA-IV

A comparative phylogenetic investigation was undertaken to describe the relationship between the PVL-negative ST22-MRSA-IV and PVL-positive ST22-MRSA-IV isolates recovered in this study and to illustrate the circulation of both PVL-positive and PVL-negative ST22-MRSA within Irish hospitals. Overall, sixty-one ST22-MRSA isolates recovered from patients (*N*=60) and a HCW (*N*=1) across nine different Irish hospitals (H1, H3, H4, H6, H7, H8, H9, H10 and H11) between 2014–2022 were identified as part of the larger CA-MRSA population structure study described above (Table 3.4). High genetic diversity was noted between these ST22 isolates, which included 25 different *spa* types and four SCC*mec* subtypes (IVa, IVc, IVh and V) (Table 3.4). The endemic PVL-negative ST22-MRSA-IV clone which predominates Irish hospitals and typically accounts for approximately 70–80% of MRSA BSIs is mainly associated with *spa* type t032 (Shore *et al*., 2014). The ST22-MRSA isolates recovered in the current study were consistent with this as the majority were also confirmed as t032 (37.7%; 23/61). Seven of the ST22 isolates were identified as PVL-positive ST22-MRSA-IVa/IVc submitted to the NMRSARL from three separate Irish hospitals between 2017–2022. The isolates were from the H1 maternity hospital and in the maternity units of two other separate Irish hospitals (H8 and H11) and were recovered from screening samples (*N*=5) and infections (*N*=2) of young individuals (aged 26–37) attending these maternity units. These PVL-positive strains were described as t005-ST22-IVc, t005-ST22-IVa, t852-ST22-IVc, t4422-ST22-IVc, t10279-ST22-IVc and t309-ST22-NT (Table 3.5), suggesting the transmission of numerous genetically distinct PVL-positive ST22-MRSA strains within the community and in healthcare environments in Ireland. Additionally, the PVL-negative HA ST22-MRSA-IV clone typically harbours a minimal number of antimicrobial resistance and virulence genes (Broderick *et al.,* 2021). By contrast, many of the PVL-positive ST22-MRSA-IV isolates investigated here carried more resistance and virulence genes than the PVL-negative strains in addition to the *pvl* genes, particularly *tst-1*, *erm*(C) and *tet*(K).

According to the cgMLST-based phylogenetic analysis performed, the PVL-negative ST22-MRSA isolates exhibited a median of 97 (average: 117; range: 1–297) allelic differences from one another, while the PVL-positive ST22-MRSA isolates exhibited a

median of 146 (average: 138; range: 43–208) allelic differences (Fig. 3.5a). The closest branching PVL-positive ST22-MRSA to the 54 PVL-negative ST22-MRSA isolates differed from these isolates by a median of 255 (average: 254; range: 233–275) allelic differences (Fig. 3.5a). To further characterise the PVL-positive ST22-MRSA and determine if these isolates belonged to widely dispersed lineages circulating internationally or if they were novel strains which emerged in Ireland independently, these NMRSARL isolates underwent phylogenetic comparison against relevant international strains from eight different countries (Table 3.5). An extensive search of publicly available literature and the NCBI/SRA/GenBank/ENA databases provided 35 different WGS sequence datasets relating to PVL-positive ST22 *S. aureus* comparator strains (21 MRSA and 14 MSSA strains). These isolates were recovered from Australia, China, Germany, India, Namibia, Nepal, UK and the USA between 1998–2019 (Table 3.5). A cgMLST analysis was performed and a MST was constructed for all 42 isolates (7 Irish and 35 international PVL-positive ST22 strains). In the cgMLST-based MST, all 42 study isolates exhibited a median of 151 (average: 149; range: 0–247) allelic differences from one another (Fig 3.5b). The seven Irish PVL-positive ST22-MRSA isolates were dispersed throughout the MST and neighboured many of the international CRFs. Notably, two Irish PVL-positive ST22-MRSA-IVc isolates recovered between 2017–2018 from H1 and H11 exhibited only 29–30 allelic differences to a cluster (C1) containing closely related (≤24 allelic differences) PVL-positive ST22-MRSA-IVc isolates recovered from the UK (*N*=4) and USA (*N*=1) between 2011–2012 (Fig. 3.5b). These findings indicate that PVL-positive ST22-MRSA strains which have achieved global dissemination are also successfully circulating within Ireland.

The 42 PVL-positive ST22 isolates investigated all had largely similar antimicrobial resistance and virulence gene profiles but exhibited distinct *spa* types and harboured three different SCC*mec* subtypes (IVa, IVc and IVd) (Table 3.5). The majority of isolates (90.5%; 38/42) carried Type B (*sak, scn, chp*) immune evasion cluster genes. A small number of isolates also harboured the toxic shock syndrome toxin-1 encoding *tst-1* gene (*N*=6), the *fus* gene (*N*=5) and the antimicrobial resistance *erm*(C) (*N*=10) and *tet*(K) (*N*=2) genes (Table 3.5).


(a)
t002
t005
t020
t022
t025
t032
t223
t309
t515
t608
t852
t1612
t2231
t2892
t2933
t2945
t3841
t4422
t4623
t10279
t13735
t14500
t17846
t19070
t19933

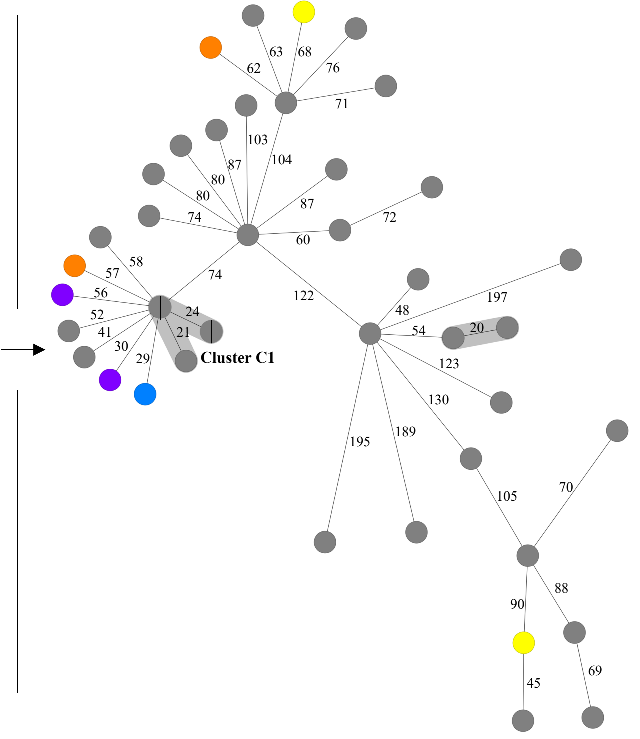

(b)
International CRFs (12 *spa* types)
Ireland (t005)
Ireland (t309)
Ireland (t4422)
Ireland (t852)
Cluster C1

Figure 3.5. Minimum spanning trees (MST) based on core-genome multi-locus sequence typing (cgMLST) analysis of ST22-MRSA isolates. **(a)** Sixty-one Irish ST22-MRSA isolates (Panton–Valentine leucocidin (PVL)-positive [*N*=7] and PVL-negative [*N*=54]) recovered as part of the CA-MRSA population structure analysis. **(b)** Seven PVL-positive ST22-MRSA Irish isolates and 35 additional epidemiologically unrelated PVL-positive ST22 international *Staphylococcus aureus* comparator reference isolates (CRFs) (21 MRSA and 14 MSSA). Closely related clusters of isolates (≤24 cgMLST allelic differences [Schürch *et al.,* 2018]) are outlined with grey shadowing. The numbers on each branch indicate the numbers of cgMLST allelic differences detected between neighbouring isolates. The epidemiological and genotypic information for each Irish isolate is shown in Table 3.4 and the CRFs in Table 3.5. (a) MST constructed from 61 Irish ST22-MRSA isolates (PVL-positive [*N*=7] and PVL-negative [*N*=54]) recovered from nine different Irish hospitals between 2014–2022. Isolates assigned to different *spa* types are indicated by separate colours. A red square around a node is used to indicate a PVL-positive isolate (b) MST constructed from the seven PVL-positive ST22-MRSA Irish isolates identified in the CA-MRSA population structure analysis and 35 additional PVL-positive international CRFs (21 MRSA and 14 MSSA). Irish isolates assigned to different *spa* types are indicated by separate colours and international strains are shaded in grey. The international strains belonged to a variety of *spa* types including t005, t13828, t1977, t1372, t1304, t852, t474, t309, t1328, t845, t1977 and t1802. ST22-MRSA-IVc isolates recovered from the UK (*N*=4) and USA (*N*=1) between 2011–2012 formed a distinct cluster (Cluster 1 [C1]).

3.3.2.6 Evidence of transmission of PVL-negative ST772-MRSA in a NICU in Ireland

To investigate the possible transmission of a PVL-negative variant of the PVL-positive ST772-MRSA-V Bengal Bay clone within a NICU of an Irish hospital over a two-week period in 2021, twelve MRSA isolates recovered between 2017–2021 were investigated using WGS (Table 3.8). Eleven of these isolates were recovered from 11 different NICU patients (aged <1) in H3 over two weeks in 2021 and one other isolate was recovered from a patient (aged 37) in the emergency department of H1 in 2017. The 11 H3 NICU isolates were PVL-negative t657/ST772-MRSA harbouring SCC*mec* V (*N*=10) or IVc (*N*=1). The remaining isolate from H1 was a PVL-positive t657/ST772-MRSA-V isolate, possibly belonging to the Bengal Bay clone. These isolates were identified as part of the larger CA-MRSA population structure study described above (Table 3.4), where close genetic relatedness between the NICU ST772 isolates was observed on the cgMLST-based MST (Fig. 3.4). As the NICU isolates were all recovered from separate patients in the same hospital unit within a relatively short time-period and appeared to be identical to one another, this prompted further analysis.

Table 3.8. Twelve ST772-MRSA Irish isolates investigated in the present study

H [*N*]	ST-SCC*mec*	No. of Isolates	Sources(*N*)	Age of patient	Recovery dates	*spa* types[b]	PVL(+/-)	AR genes*(N)*	Virulence factor genes	IEC
H3 [11]	ST772-MRSA-V	10	NICU [10]	<1 [10]	2021 [10]	t657 [10]	PVL- [10]	*blaZ* [10], *ccrAA/C* [10], *mecA* [10], *lmrP* [10], *aac-aphD* [10], *aphA3* [10], *mphC* [10], *ugpQ* [10], *fosB* [10], *mprF* [10], *msrA* [10], *sdrM* [10]	*aur* [10], *ebpS* [10], *eno* [10], *hl* [10], *hla* [10], *hlgA/B/C* [10], *lukX/Y* [10], *sei* [10], *sec* [10], *sspB/P* [10]	H (*scn*) [10]
	ST772-MRSA-IVc	1	NICU	<1	2021	t657	PVL-	*blaZ*, *ccrAA/C*, *mecA*, *lmrP*, *aac-aphD*, *aphA3, mphC*, *ugpQ*, *fosB, mprF*, *msrA, sdrM*	*aur*, *ebpS*, *eno*, *hl*, *hla*, *hlgA/B/C*, *lukX/Y*, *sei*, *sec*, *sspB/P*	H (*scn*)
H1 [1]	ST772-MRSA-V	1	ED	37	2017	t657	PVL+	*blaZ*, *ccrAA/C*, *mecA*, *lmrP*, *aac-aphD*, *aphA3, mphC*, *ugpQ*, *fosB, mprF*, *msrA, sdrM*	*aur*, *ebpS*, *eno*, *hl*, *hla*, *hlgA/B/C*, *lukF/S-PV*, *lukX/Y*, *sei*, *sec*, *sspB/P*	*sea, scn*

Abbreviations: N, number; H, hospital; ST, sequence type; NICU, neonatal intensive care unit; ED, emergency department; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; PVL, Panton-Valentine leukocidin; +, positive; -, negative; IEC, immune evasion cluster.

By cgMLST, the PVL-negative NICU isolates exhibited a median of 0 (average: 0; range: 0–1) allelic differences from one another and between 73–76 allelic differences to the PVL-positive H1 isolate (Fig. 3.6a). By wgMLST, the NICU isolates exhibited a median of 3 (average: 3; range: 1–9) allelic differences from one another and between 96–106 allelic differences to the PVL-positive H1 isolate (Fig. 3.6b), indicating that there was likely one transmission event or outbreak between the patients in the NICU of H3. Genomic comparison of the PVL-negative t657/ST772-MRSA-IVc/V isolates recovered in this study against the well-established PVL-positive t657/ST772-MRSA-V Bengal Bay clone revealed high genetic similarities between these strains, despite the lack of *pvl* genes in the Irish study isolates. Analysis of the typical position of the PVL-encoding bacteriophage integration site revealed a complete absence of the phage in the PVL-negative NICU isolates, although more thorough investigation is required. Comparison of the PVL-negative H3 NICU isolates with the PVL-positive H1 isolate in this study revealed that the H1 isolate differed from the NICU isolates through carriage of the *pvl* genes and an additional immune evasion cluster gene. All isolates carried the staphylococcal complement inhibitor (*scn*) gene but only the PVL-positive isolate harboured the staphylococcal enterotoxin A (*sea*) gene. These preliminary findings suggest that the emergence of this PVL-negative t657/ST772-MRSA lineage is worth exploring further to determine its origins, route of transmission into hospitals and its relationship to the Bengal Bay clone which is known to be circulating widely across Irish hospitals and community settings. Further analysis is currently ongoing and involves collation of an extensive collection of similar PVL-positive and PVL-negative ST772 isolates from numerous disparate regions.

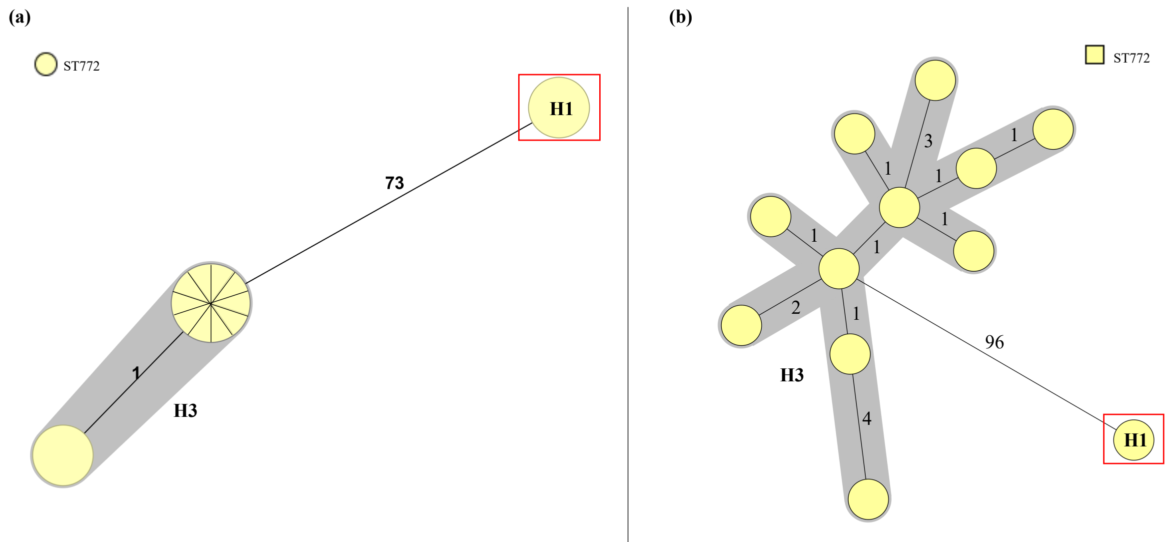


Figure 3.6. Minimum spanning trees (MST) based on core-genome multi-locus sequence typing (cgMLST) analysis and whole-genome multi-locus sequence typing (wgMLST) analysis of the 12 Irish ST772-MRSA isolates (Panton–Valentine leucocidin (PVL)-positive [*N*=1] and PVL-negative [*N*=11]) recovered as part of the CA-MRSA population structure analysis. Hospital association is indicated (H1 and H3). Closely related clusters of isolates (≤24 cg/wgMLST allelic differences [Schürch *et al*., 2018]) are outlined with grey shadowing. The numbers on each branch indicate the numbers of cg/wgMLST allelic differences detected between neighbouring isolates. The epidemiological and genotypic information for each isolate investigated is shown in Table 3.4 and 3.7. **(a)** cgMLST-based MST constructed from 12 Irish ST772-MRSA isolates (PVL-positive [*N*=1] and PVL-negative [*N*=10]) recovered from two different Irish hospitals between 2017–2021. A red square around a node is used to indicate a PVL-positive isolate. **(b)** wgMLST-based MST constructed from 12 Irish ST772-MRSA isolates (PVL-positive [*N*=1] and PVL-negative [*N*=10]) recovered from two different Irish hospitals between 2017–2021. A red square around a node is used to indicate a PVL-positive isolate.

3.4 Discussion

This study provided insights into the epidemiology of CA-MRSA in Ireland and confirmed the transmission of PVL-positive CA-MRSA into healthcare settings and subsequent association of these lineages with infection outbreaks. Multiple repeated introductions of PVL-positive CA-MRSA, specifically ST8-MRSA-IV (USA300 clone), ST772-MRSA-V (Bengal Bay clone) and ST88-MRSA-IV ('African' clone) have all previously been identified in Ireland, as well as association of these lineages with outbreaks in nosocomial settings (Brennan *et al.,* 2012; Earls *et al.,* 2018; Shore *et al.,* 2014). Many of these lineages are highly transmissible clones with the capability to spread widely and possibly displace predominant strains, making the high prevalence of PVL-positive CA-MRSA lineages observed in the current study a major concern. The PVL-negative ST22-MRSA-IVa lineage has maintained its predominance in Irish hospitals since 2002, but periodic replacement of dominant clonal types has occurred several times previously in Ireland (Shore *et al.,* 2012). With the increased diversity in lineages being introduced into hospitals, potentially from the community as observed in the current study, another displacement could potentially occur in the near future. Alongside the prevalent ST1, ST5 and ST8 lineages reported in this study, genetically diverse PVL-positive ST22-MRSA and PVL-negative ST772-MRSA strains were also described. While the overall proportion of these strains were quite low (2% for PVL-positive ST22; 3% for PVL-negative ST772), the circulation of these genetically distinct strains is still concerning. Reports of PVL-positive ST22-MRSA-IV associated with both healthcare and community outbreaks is well-documented, particularly in India where it now predominates together with the CA-MRSA ST772 Bengal Bay clone within healthcare environments (Shore *et al.,* 2014). In Ireland, the circulation of novel distinct PVL-positive ST22 CA-MRSA lineages, separate to the highly prevalent PVL-negative HA ST22-MRSA, has been linked with outpatient clinics and NICUs (Broderick *et al.,* 2021). Similarly, the Bengal Bay clone has also been associated with colonisation and infection of both HCWs and NICU patients in Ireland (Brennan *et al.,* 2012). Characterisation of these lineages reveal that importation into Ireland may have occurred on a number of different occasions. Introduction of these genetically distinct lineages which were originally identified from disparate geographical regions into Ireland suggests that surveillance of patient travel history and family background should be at the forefront of IPC measures to combat MRSA spread, alongside targeted patient screening.

Earls *et al.,* 2017, also recently reported the emergence and extensive spread of a novel multidrug resistant t127/ST1-MRSA-IV within healthcare environments and the community in Ireland (Earls *et al.,* 2017). The proportion of this t127 CA-MRSA lineage identified at the NMRSARL gradually increased from 1–7% between 2010–2015 (Earls *et al.,* 2017). In the current study, PVL-positive t127 ST1-MRSA isolates which caused outbreaks within two separate families associated with the same hospital several years apart were identified. This demonstrated the involvement of intra-familial transmission in the spread of MRSA in the community. A previous report suggested that intra-familial transmission of MRSA is quite common and can result in two-thirds (67%) of all household contacts of an index carrier becoming colonised from the same strain (Mollema *et al.,* 2010). Furthermore, recent travel history recorded for some members of Family 2 indicated that they had travelled to the Middle East in the past, which suggests possible importation of these strains into Ireland and subsequent spread within the family. Additionally, the involvement of HCWs in the spread of CA-MRSA within healthcare settings is becoming increasingly recognised (Earls *et al.,* 2017). The current study revealed the presence of two distinct outbreaks involving PVL-positive ST5-MRSA and ST88-MRSA lineages which occurred simultaneously within one hospital. One of the ST88 isolates was recovered from a HCW within this hospital. The transfer of staff and patients between different healthcare facilities has been suggested as a likely contributor to the successful transmission of CA-MRSA in Ireland, although no evidence of this was noted in the present study where outbreaks were specific to separate hospitals (Brennan *et al.,* 2012; Earls *et al.,* 2017; L. Wang *et al.,* 2012).

Traditionally, resistance to multiple classes of clinically relevant antibiotics is not a common characteristic of CA-MRSA. This is no longer the case as MDR is now increasingly observed in many CA-MRSA strains including in PVL-positive populations across Asia, Europe, the USA and Australia (Brennan *et al.,* 2012; Earls *et al.,* 2017; Macedo-Vinas *et al.,* 2013; Wang *et al.,* 2012). In the present study, although the majority of the population exhibited resistance to only a limited number of the antibiotic classes tested, MDR was detected in 37% of the PVL-positive CA-MRSA outbreak isolates investigated. Acquisition of additional antimicrobial resistance determinants by CA-MRSA strains, including the well-established ST8 USA300 through MGEs has been suggested as a potential factor aiding CA-MRSA's successful invasion of healthcare settings (Lindsay *et al.,* 2012). This is of major concern as it limits the treatment options

available for infected patients and greatly impacts their clinical outcomes. Phenotypic resistance to tetracycline, ciprofloxacin and erythromycin was detected in 9%, 13% and 46% of isolates investigated as part of the PVL-positive CA-MRSA outbreak study, respectively (Table 3.1). Furthermore, the PVL-positive t127 and PVL-negative t267 isolates investigated in the present study all exhibited phenotypic resistance to fusidic acid and carried the *fus* gene. Although systemic use of fusidic acid in Ireland has significantly decreased, it is still often prescribed as a topical treatment for SSTIs (Earls *et al.,* 2017). Overall, this demonstrates the advantage of epidemiological investigations such as this one in informing antimicrobial treatment options and implementing infection prevention and control (IPC) strategies.

Outbreak studies were often limited to characterisation of strains by *spa* typing and traditional MLST approaches (Brennan *et al.,* 2012; Broderick *et al.,* 2021). These techniques have lower discriminatory power in comparison to WGS-based tools, and are inadequate for discerning differences among closely related strains, particularly in outbreak situations. Using WGS, the current study confirmed outbreaks caused by distinct PVL-positive CA-MRSA lineages (ST8-MRSA-IVa, ST88-MRSA-V and ST5-MRSA-IVc) within separate Irish hospitals attending to maternity and paediatric patients. Transmission of CA-MRSA strains within NICUs and among pregnant patients is of concern, and outbreaks of this nature within such settings have been increasingly reported in recent years (Gould *et al.,* 2009; Sanchini *et al.,* 2013; Sowash and Uhlemann, 2014). In the present study, many of the outbreak isolates were recovered from patients within the same hospital wards, from HCWs and also from family clusters, indicating that there are currently numerous routes for CA-MRSA importation into hospital settings. The majority of these CA-MRSA outbreak isolates belonged to strains circulating locally in the community which were then imported into hospitals. The family cluster outbreaks also revealed an alternate route of transmission with the importation of international strains by travel resulting in household spread, followed by introduction into hospitals.

The current study demonstrated the high resolution offered by WGS over conventional molecular typing for investigation of outbreaks and transmission of MRSA strains both within and outside healthcare settings. In conclusion, although this study successfully highlights the changing epidemiology of MRSA in Ireland and uses WGS to supplement the currently limited data on CA-MRSA spread, the actual burden of this pathogen could

be significantly worse. Some limitations to the study include reliance on only those isolates submitted to the NMRSARL and also lack of sufficiently detailed epidemiological information on patient travel history and family background. Additionally, although maternity hospital patients are thought to be reflective of the general MRSA population, it is important to note that there may be some level of disparity between strains circulating in maternity settings and those within the general community. Addressing these limitations will positively impact MRSA surveillance investigations and inform more effective IPC approaches.

Chapter 4

An emerging Panton-Valentine leukocidin-positive CC5-MRSA-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by whole-genome sequencing

4.1 Introduction

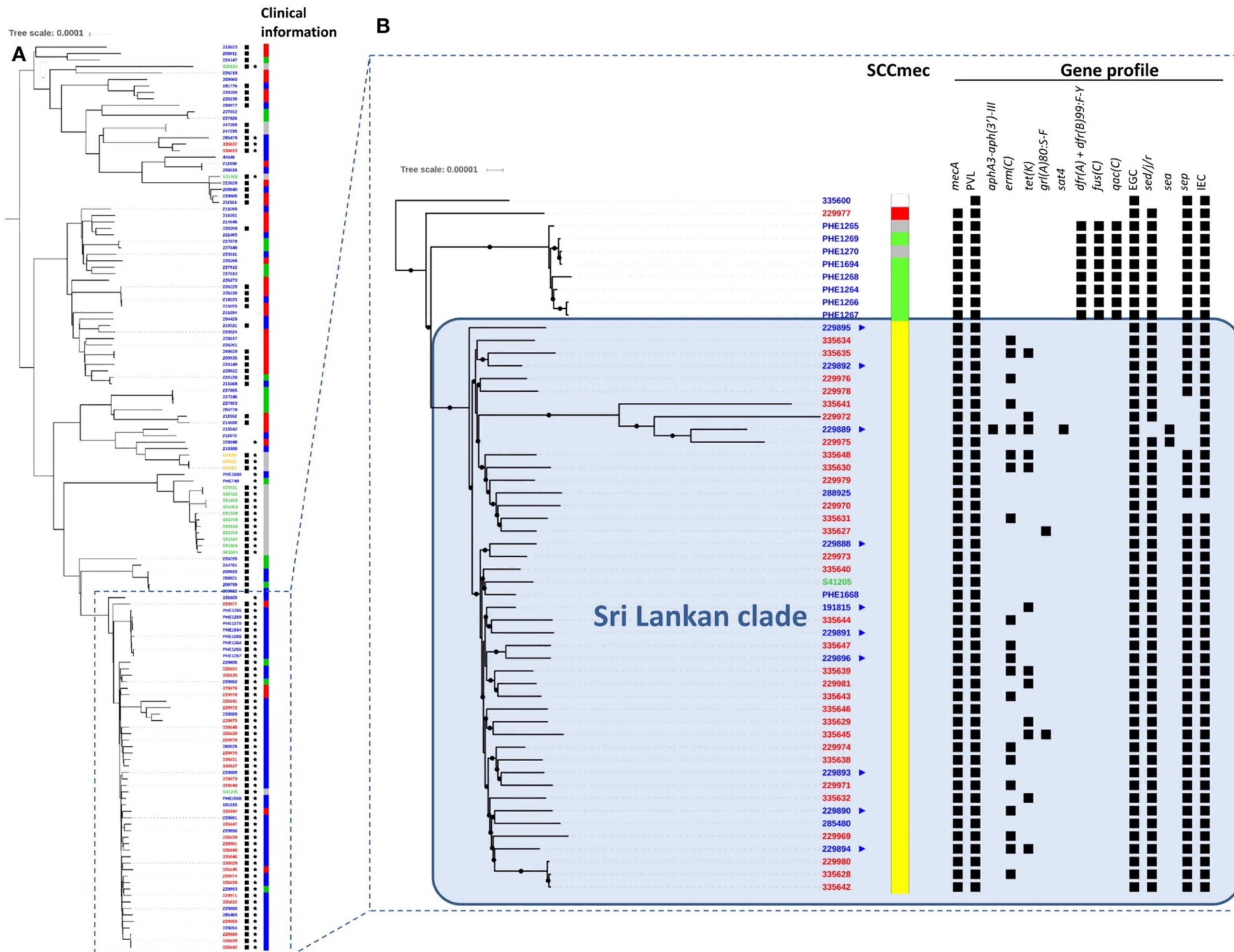
4.1.1 PVL-positive MRSA in Ireland

Between 1999 and 2005, the overall prevalence rate reported for PVL-positive MRSA in Ireland was approximately 1.8% and the predominant lineages identified were ST5, ST8, ST22, ST30, ST80 and ST154, all harbouring SCC*mec* type IV (Shore *et al.,* 2014). Since then, the proportion of PVL-positive MRSA isolates submitted to the NMRSARL annually has increased significantly (NMRSARL, 2020; Shore *et al.,* 2014). According to recent NMRSARL reports, the prevalence of PVL-positive isolates increased from 0.2% in 2002 to 8.8% in 2011 and 16.8% in 2022 (Fig. 3.1), with the most frequently recognised STs including ST1, ST5, ST8 and ST30 which typically harbour SCC*mec* types IV or V (NMRSARL, 2021; Shore *et al.,* 2014). These PVL-positive lineages now account for approximately 35% of all circulating strains in Ireland (NMRSARL, 2021; Monecke *et al.,* 2011).

4.1.2 Identification of a novel PVL-positive ST5-MRSA clone in Ireland

Chapter 3 of this study described the recent emergence of a PVL-positive CC5/ST5-MRSA lineage harbouring the SCC*mec* type IVc element in an Irish hospital. This CC5 lineage was associated with a distinct MRSA outbreak in the maternity unit of a regional Irish hospital (H3) which involved 13 separate patients over a 15-month period between 2018 and 2020. Prior to this, similar isolates had been identified within a major teaching hospital in the rural North-Central province of Sri Lanka in 2014, as well as in Australia and the UK and were described by McTavish *et al*., 2019 as 'Sri Lankan clone' MRSA. The isolates were recovered over a four-month period as part of a general population study of MRSA causing clinical infections in Sri Lanka. The majority of PVL-positive isolates recovered in the study (56/62; 90.3%) belonged to the dominant CC5/ST5-MRSA-IVc Sri Lankan clone. To confirm whether this PVL-positive CC5 lineage was genotypically diverse or part of a single circulating clone, a WGS-based phylogenetic investigation (Fig. 4.1) was undertaken by McTavish *et al*. The analysis, which also included representative previously sequenced PVL-positive ST5-MRSA-IVc isolates from the UK and Australia revealed that the CC5/ST5-MRSA-IVc isolates formed a single phylogenetic clade (Fig 4.1), suggesting widespread geographical circulation of this clone. The majority of the UK ST5-MRSA-IVc samples (10/13; 76.9%) in the Sri Lankan clade were associated with patients with known travel links to Sri Lanka. Six

related *spa* types were associated with the Sri Lankan clone including t010, t045, t1062, t5490, t7342 and t002; with the latter being the predominant *spa* type (50/56; 89.3%).


Clinical information
A
Tree scale: 0.0001
B
SCCmec
Gene profile
mecA
PVL
aphA3-aph(3')-III
erm(C)
tet(K)
grl(A)80:S-F
sat4
dfr(A) + dfr(B)99:F-Y
fus(C)
qac(C)
EGC
sed/j/r
sea
sep
IEC
Tree scale: 0.00001
335600
229977
PHE1265
PHE1269
PHE1270
PHE1694
PHE1268
PHE1264
PHE1266
PHE1267
229895
335634
335635
229892
229976
229978
335641
229972
229889
229975
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229970
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335627
229888
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229896
335639
229981
335643
335646
335629
335645
229974
335638
229893
229971
335632
229890
285480
229969
229894
229980
335628
335642
Sri Lankan clade

Figure 4.1. Phylogenetic investigation performed by McTavish *et al*. 2019, on CC5 isolates from Sri Lanka, England, Australia, and Argentina to describe the Sri Lankan clone. Phylogeny inferred by maximum likelihood analysis. Country of origin denoted by colour of sample name: blue, England; red, Sri Lanka; green, Australia; yellow, Argentina. **(A)** Unrooted phylogenetic tree indicating relationships between international CC5 *S. aureus* based on SNP analysis of whole genome sequences. Clinical information relating to each isolate is also shown (red, invasive; blue, skin and soft tissue infection; green, screening/carriage sample; grey, unknown). **(B)** The Sri Lankan clade. Triangle indicates UK patients with known links to Sri Lanka. SCC*mec* types: red, IV-a; yellow, IV-c; green, VI; grey, NT. In gene profile section, ■ indicates presence of gene. Image from McTavish *et al*., 2019.

Where origin of the sample was recorded, the majority of the Sri Lankan clone isolates recovered in the study were primarily associated with community and hospital-onset SSTIs (42/50; 84%), with the remainder from invasive infections including bacteraemia, empyema and osteomyelitis. All but one PVL-positive ST5-MRSA-IVc isolate harboured the *sed/sej/ser* enterotoxin genes, which are typically harboured on plasmids (Fig. 4.2). Greater variability was observed in carriage of genes encoding antimicrobial resistance, including *erm*(C) and *tet*(K), which suggests dynamic loss/acquisition of mobile genetic elements within this clone. A chromosomal mutation associated with quinolone resistance (*grlA* 80:S-F) was also noted sporadically. Similarly, a recent genomic analysis of MRSA circulating in the UAE by Senok *et al.* 2020, also revealed the presence of the PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone in secondary and tertiary care facilities, further demonstrating wider geographical spread of this clone. The UAE ST5-MRSA-IVc isolates also exhibited two distinct profiles based on the presence or absence of the *sed/sej/ser* enterotoxin genes (Fig. 4.2).

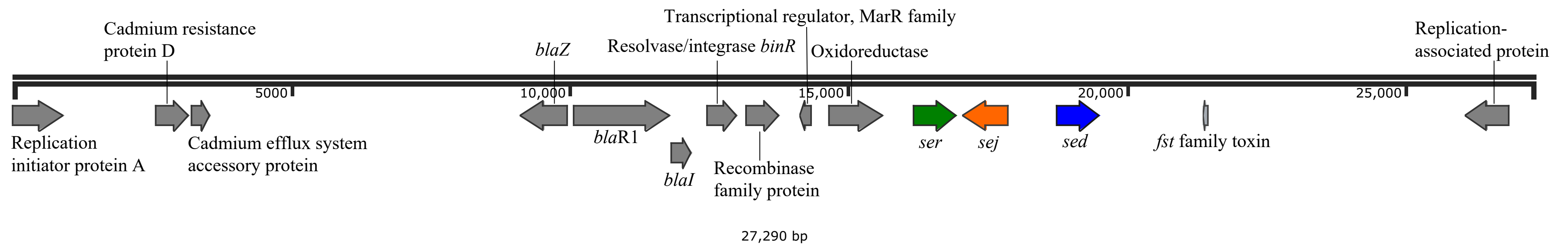

Figure 4.2. *Staphylococcus aureus* pSM31 plasmid present in a PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolate M4761 carrying the *ser, sej, sed* enterotoxin genes and β-lactamase genes including *blaZ, bla*R1 and *blaI.* Isolate sequence underwent hybrid assembly following long-read and short-read sequencing. The hybrid assembled genome was annotated using RAST v2.0 (https://rast.nmpdr.org) and visualised using SnapGene v6.0.6 (https://www.snapgene.com).

4.1.3 Prevalence of CC5/ST5-MRSA in Ireland and internationally

Clonal complex 5 MRSA includes several epidemic clones associated with ST5 responsible for community and healthcare-associated infections globally. This includes the well dispersed USA800 and USA100 clones (Breurec *et al.,* 2011). USA800 is a PVL-positive ST5-MRSA clone (also known as the Paediatric clone) which was first detected in neonatal patients in a paediatric hospital in Portugal in the late 1990s, before gradually spreading across other parts of Europe, including Ireland (Shore *et al.,* 2005) and also into North/South America (Sá-Leão *et al.,* 1999). Many isolates belonging to this clone are known to harbour a SCC*mec* structural variant similar in size to type IV but with a novel *ccrAB* allotype, termed SCC*mec* type VI (Conceição *et al.,* 2010; Oliveira *et al.,* 2006). This ST5-MRSA clone now circulates widely within the community and in hospital settings and is associated with a variety of clinical manifestations including severe nosocomial infections (de Miranda *et al.,* 2007). Although PVL-negative, the USA100/ST5-MRSA-II clone (also known as the New York/Japan clone) is another clinically relevant ST5 lineage which has achieved global dissemination (Grundstad *et al.,* 2019). USA100 isolates typically exhibit a high level of antimicrobial resistance and are often associated with a variety of invasive infections across all patient populations (Grundstad *et al.,* 2019). In Ireland, ST5-MRSA-II has been a common clonal type since the early 1990s with numerous variants of the clone identified over the years (Shore *et al.,* 2005). This includes Irish AR07.3, AR07.4 and AR11, which all exhibit distinct antimicrobial resistance profiles (Shore *et al.,* 2005). Additionally, ST5-MRSA-II isolates are typically associated with *spa* types t002 and t003 (Monecke *et al.,* 2011). In Ireland, ST5-MRSA-II assigned to *spa* type t045 have also been reported (Monecke *et al.,* 2011). Spread of MRSA through travel and migration is well-described (McTavish *et al.,* 2019; Shore *et al.,* 2014), and can explain the diversity of clones imported into Ireland. The significant increase observed in the proportion of PVL-positive MRSA including increased incidences of ST5-MRSA in recent years, has primarily been attributed to an increase in the number of outbreaks and infection clusters in Irish community and hospital settings (NMRSARL, 2021). This is evidenced by the multiple distinct outbreaks within a short period of time described in Chapter 3 of this study.

4.1.4 Surveillance of the emerging PVL-positive Sri Lankan clone

The importation of the PVL-positive ST5-MRSA-IVc Sri Lankan clone into Ireland as well as its subsequent association with infection outbreaks within Irish nosocomial settings is of concern. Additionally, the lower fitness cost imposed by carriage of a smaller SCC*mec* element (Type IV) could potentially allow for stable populations of this CA-MRSA clone to arise and disseminate widely across community and nosocomial settings (Breurec *et al.,* 2011). Chapter 3 of this study focused only on localised investigation of the Sri Lankan clone within a specific hospital setting in Ireland. Similarly, international investigations on this clone have also only provided country-specific genomic characterisation of the clone (McTavish *et al.,* 2019; Senok *et al.,* 2020). The initial genomic investigation of the Sri Lankan clone from Sri Lanka, the UK and Australia by McTavish *et al*., 2019, highlighted the need for investigation of a larger collection of isolates from numerous disparate geographical regions in order to accurately elucidate the origins and dissemination of the Sri Lankan clone. Consequently, the present study sought to further investigate the Sri Lankan clone using a comprehensive collection of PVL-positive ST5-MRSA-IVc isolates from Irish hospital and community settings in comparison with the previously reported Sri Lankan clone (McTavish *et al.,* 2019) and similar international isolates to determine the clone's global distribution, diversity and population structure for the first time using WGS.

4.2 Materials and Methods

4.2.1 Isolate collection

Two hundred and eighty-five isolates recovered from 15 different countries between 2003 and 2022 were investigated including: (i) PVL-positive CC5/ST5-MRSA-IVc isolates (2005–2022) from 12 countries across Europe, Asia, Australia and the Middle East similar to and including 46 previously described Sri Lankan clone isolates (McTavish *et al.,* 2019) (ii) PVL-negative t311/ST5-MRSA-V isolates recovered from five separate Irish hospitals between 2015–2019 and (iii) PVL-positive and PVL-negative comparator CC5/ST5-MRSA–I/II/IVa/IVc/IVg/V isolates recovered between 2003–2021. Isolates were stored as described in Chapter 2, Section 2.2.1. A detailed breakdown of the isolate collection is provided below.

4.2.1.1 Irish study isolates

Fifty-six MRSA isolates submitted to the NMRSARL between 2013–2022 were investigated in this part of the study. This included 14 previously described PVL-positive ST5/t002-MRSA-IVc outbreak-associated isolates recovered from a maternity ward (*N*=9), the special care baby unit (SCBU) (*N*=3), the outpatient department (*N*=1) and the paediatric emergency department (*N*=1) of a regional Irish maternity hospital (H3) between 2018–2020 (see Chapter 3). These isolates were suspected to be outbreak-associated due to the short 15-month period between isolate recovery in the same hospital, and subsequently underwent confirmatory WGS analysis. The isolates were genotypically similar to Sri Lankan clone isolates which were previously described by McTavish *et al.,* 2019. A search of the NMRSARL collection for PVL-positive t002/ST5-MRSA-IVc and related *spa* types revealed 16 additional isolates recovered between 2013 and 2022 (Table 4.1). This included two more recent (2021) patient isolates from the same maternity unit and 14 patient isolates from nine other hospitals across Ireland (H2, H4, H9, H11, H13–H15 and H17–H18).

Table 4.1. Antimicrobial resistance and virulence-associated gene profiles of 214 PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates and 52 additional PVL-positive [*N*=29] and PVL-negative [*N*=23] CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates investigated

Country	Isolates [*N*]	Year(s) of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[a] [*N*]	PVL (+/-)	IEC Type [*N*]	Reference
Algeria	Comparator [1]	2003	t450-ST5-IVa	*aadD, erm*(C), *fosB, lmrP, mprF, tet*(M), *vga*(A), *sdrM*	+	B	This study
Australia	Sri Lankan clone [1]	2015	t002-ST5-IVc	*blaZ, fosB, lmrP, sdrM*	+	G	McTavish *et al.*, 2019
Czech Republic	Sri Lankan clone [6]	2018–2021	t002-ST5-IVc	*blaZ* [4], *fosB [*6], *lmrP [*5], *sdrM* [6], *mprF* [6], *erm*(C) [1]	+	G [4] E [1] Novel type 3 (*sak, sep*) [1]	This study
	Comparators [2]	2019–2021	t002-ST5-IVa [1] t002-ST5-II [1]	*aadD* [1], *blaZ* [2], *erm*(A) [1], *fosB* [2], *kdpA/B/C/D/E* [1], *lmrP* [2], *mprF* [2], *sdrM* [2], *xylR* [1]	-	G [1] B [1]	
Denmark	Sri Lankan clone [66]	2007–2021	t002-ST5-IVc	*blaZ* [61], *fosB [*65], *lmrP* [65], *mprF* [65], *sdrM* [66], *erm*(C) [31], *tet*(K) [3], *mupA* [3], *qacA* [3], *qacC* [1], *cat* [1]	+	G [59] F [7]	This study
	Comparators [10]	2013–2015	t002-ST5-IVa [2] t002-ST5-V [8]	*blaZ [*9], *fosB [*10], *lmrP* [10], *mprF* [10], *sdrM* [10], *aacA-aphD* [7], *erm*(C) [2], *tet*(K) [1]	+ [7] - [3]	G [8] F [1] B [1]	
Germany	Sri Lankan clone [20]	2011–2019	t002-ST5-IVc [15] t535-ST5-IVc [2] t579-ST5-IVc [1] ND-ST5-IVc [2]	*blaZ* [18], *erm*(C) [11], *fosB* [20], *lmrP* [20], *mprF* [20], *sdrM [*19], *tet*(K) [1], *qacA* [1], *msr*(A) [1]	+	G [16] E [1] Novel type 1 (*sep* only) [1] Novel type 2 (*sak, scn, sea, sep*) [1]	This study
	Comparators [2]	2014–2017	t105-ST5-IVc	*blaZ* [1], *fosB* [1], *lmrP [*1], *mprF [*2], *sdrM* [1]	+	B	

Table 4.1 continued overleaf

Country	Isolates [*N*]	Year(s) of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[a] [*N*]	PVL (+/-)	IEC Type [*N*]	Reference
Ireland	Sri Lankan clone [30]	2013–2022	t002-ST5-IVc	*blaZ* [29], *fosB* [30], *lmrP* [30], *mprF* [30], *sdrM* [30], *erm*(C) [5]	+	G [29] None [1]	This study
	Comparator [17]	2013–2019	t002-ST5-I [1] t002-ST5-IVa [2] t002-ST5-IVc [3] t002-ST5-IVg [2] t311-ST5-V [9]	*blaZ* [13], *erm*(C) [10], *fosB* [17], *lmrP* [17], *mprF [*17], *sdrM* [17], *fusC* [10], *fexA* [1], *aadD [*1], *qacA* [1], *merA* [1]	+ [2] - [15]	G [2] F [2] B [3] E [9] Novel type 1 (*sep* only) [1]	
Kuwait	Sri Lankan clone [1]	2013	t002-ST5-IVc	*blaZ, erm*(C), *fosB, lmrP, mprF, sdrM*	+	G	This study
	Comparator [1]	2013	t002-ST5-IVa	*blaZ, erm*(C), *fosB, lmrP, mprF, sdrM*	+	G	
Norway	Sri Lankan clone [24]	2007–2021	t002-ST5-IVc [23] t1062-ST5-IVc [1]	*blaZ* [23], *fosB* [24], *lmrP* [24], *mprF* [24], *sdrM* [24], *erm*(C) [9], *tet*(K) [2], *vga*(A) [1]	+	G [23] Novel Type 2 (*sak, scn, sea, sep*) [1]	This study
	Comparators [12]	2003–2020	t311-ST5-IVa [4] t311-ST5-IVc [2] t002-ST5-IVa [3] t105-ST5-IVc [1] t3089-ST5-IVa [1] t442-ST5-V [1]	*blaZ* [12], *fosB* [12], *lmrP* [12], *mprF* [12], *sdrM* [12], *erm* [C] [1], *tet*(K) [1], *aacA-aphD* [2], *dfrA [*2], *tet*(M) [2], *aphA3* [3], *mph*(C) [1], *msr*(A) [1], *sat* [3], *qacC* [1]	+ [11] - [1]	G [3] B [5] A [4]	
Saudi Arabia	Sri Lankan clone [4]	2010–2017	t002-ST5-IVc	*blaZ* [3], *fosB* [4], *lmrP* [4], *mprF* [4], *sdrM* [4], *erm*(C) [2], *aphA3* [1], *sat* [1]	+	G [4]	This study
	Comparator [1]	2010	t311-ST5-IVa	*blaZ, fosB, lmrP, mprF, sdrM*	-	B	
Senegal	Comparators [2]	2007	t311-ST5-IVa	*aacA-aphD* [1], *aadD* [1], *blaZ* [1], *dfrA* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *qacC* [1], *tet*(M) [2], *sdrM* [2]	+	B [2]	This study

Table 4.1 continued overleaf

Country	Isolates [*N*]	Year(s) of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[a] [*N*]	PVL (+/-)	IEC Type [*N*]	Reference
Slovakia	Comparator [1]	2020	t002-ST5-IVc	*blaZ, fosB, lmrP, mprF, sdrM*	-	B	This study
Sri Lanka	Sri Lankan clone [33]	2014	t002-ST5-IVc [21] t010-ST5-IVc [1] t045-ST5-IVc [2] t062-ST5-IVc [4] t1062-ST5-IVc [1] ND-ST5-IVc [4]	*blaZ* [33], *fosB* [33], *lmrP* [33], *mprF* [33], *sdrM* [33], *erm*(C) [14], *tet*(K) [4]	+	G [31] Novel type 1 (*sep* only) [1] Novel Type 2 (*sak, scn, sea, sep*) [1]	McTavish *et al.*, 2019
Sweden	Sri Lankan clone [2]	2005–2009	t002-ST5-IVc	*blaZ* [2], *erm*(C) [1], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2], *tet*(K) [1]	+	G [2]	This study
United Arab Emirates	Sri Lankan clone [15]	2017–2019	t002-ST5-IVc [12] t010-ST5-IVc [1] t045-ST5-IVc [1] t306-ST5-IVc [1]	*blaZ* [15], *erm*(C) [9], *fosB* [15], *lmrP* [17], *mprF* [17], *sdrM* [17]	+	G [15]	This study
	Comparator [3]	2018	t105-ST5-IVc [2] t002-ST5-IVa [1]	*blaZ* [1], *erm*(C) [1], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G [1] B [2]	
United Kingdom	Sri Lankan clone [12]	2005–2015	t002-ST5-IVc [5] t062-ST5-IVc [2] ND-ST5-IVc [5]	*blaZ* [12], *fosB* [12], *ImrP* [12], *mprF* [12], *sdrM* [12], *erm*(C) [4], *tet*(K) [2], *sat* [1]	+	G [11] D [1]	McTavish *et al.*, 2019

[a] Genotypic information was extracted from whole-genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) and *S. aureus* Genotyping Kit 2.0 (Abbott [Alere]) microarray technology (Monecke *et al*., 2016).

Abbreviations: ND, not determined – isolates not available and *spa* types could not be determined using *in-silico* techniques with the available genomic sequence data; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; PVL, Panton-Valentine leukocidin; +, positive; -, negative; IEC, immune evasion cluster; *scn*; staphylococcal complement inhibitor, *sea*; staphylococcal enterotoxin a gene, s*ep*, staphylococcal enterotoxin p gene; *sak*, staphylokinase gene.

Surveillance of ST5-MRSA submitted to the NMRSARL for investigation also revealed a noticeable increase in the number of PVL-negative ST5-MRSA isolates during the same period of the protracted PVL-positive t002/ST5-MRSA-IVc outbreak in H3. Notably, there was an increase in the number of PVL-negative t311/ST5-MRSA-V isolates submitted to the NMRSARL from five separate Irish hospitals. Due to the close genetic relationship between *spa* type t002 (repeat succession: 26–23–17–34–17–20–17–12–17–16) and *spa* type t311 (repeat succession: 26–23–17–34–20–17–12–17–16), which differ by only one *spa* repeat and association of both *spa* types with ST5, a comparative investigation was undertaken to illustrate the circulation of both PVL-positive and PVL-negative ST5-MRSA within Ireland. Twenty-six PVL-negative t311/ST5-MRSA-V isolates recovered between 2015–2019 from H1, H3, H6, H13 and H14 were investigated as part of this study within the context of the fourteen previously described PVL-positive t002/ST5-MRSA-IVc outbreak isolates recovered between 2018–2020 from H3 (Table 4.2).

4.2.1.2 Irish comparator reference isolates

Nineteen comparator NMRSARL isolates (two PVL-positive ST5-MRSA-IVa, one PVL-negative ST6045-MRSA-V and 16 PVL-negative ST5-MRSA-I/IVc/IVg/V) recovered from nine hospitals (H2–H4, H9, H13, H19–H22) between 2013 and 2019 were investigated as part of the current study (Table 4.1 and 4.2).

4.2.1.3 International study isolates

Additional PVL-positive ST5-MRSA-IVc clinical isolates or WGS datasets from disparate geographical locations were sought for comparison to the Irish PVL-positive ST5-MRSA-IVc isolates. Contact with international collaborators, a literature search (Table 4.3) and an extensive search of the NCBI, SRA, GenBank and ENA databases using the following search terms: 'ST5', 'CC5', '*spa* type t002', 'PVL-positive', '*lukF-pv', 'lukS-pv'*, 'MRSA' and 'SCC*mec* IVc' yielded 184 international strains. This included clinical isolates (*N*=56) and WGS datasets (*N*=82) from international collaborators as listed in Table 4.1 and summarised below. From the literature search, 46 PVL-positive ST5-MRSA-IVc Sri Lankan clone isolates were recovered (Table 4.3).

Table 4.2. Molecular characterisation, antimicrobial resistance and virulence gene profiles of the 26 t311, 14 t002 ST5-MRSA and seven comparator isolates investigated in the present study

spa-ST-SCC*mec*	No. of isolates investigated [*N*]					
	Isolates [*N*]	Source[a]	Antibiotic resistance	AR genes[a]	PVL(+/-)	IEC Type
(a) Study isolates						
t002/ST5-MRSA-IVc	14	H3	Ap [14]	*blaZ, fosB, lmrP, mecA, mprF* [14]	PVL+ [14]	G [14]
t311/ST5-MRSA-V	26	H1 [1] H3 [1] H6 [4] H13 [11] H14 [9]	Ap, Fd, Cp, Tp [8] Ap, Fd, Cp, Er, Tp [14] Ap, Fd, Te, Rf [2] Ap, Fd, Er, Te, Rf [2]	*blaZ, fosB, fusC, ImrP, mecA, erm*(C), *mprF, sdrM* [13] *blaZ, fosB, fusC, ImrP, mecA, mprF, sdrM* [8] *fosB, fusC, ImrP, mecA, mprF sdrM, tet*(M) [3] *blaZ, fosB, fusC, ImrP, mecA, mprF, sdrM, tet* [M], msr*A* [1] *blaZ, fosB, fusC, ImrP, mecA, erm*(C), *mprF, sdrM, qac*(A) [1]	PVL- [26]	E [22] B [4]
(b) Comparator isolates						
t311/ST6045-MRSA-V	1	H13	Ap, Fd, Cp, Er, Tp	*blaZ, fosB, fusC, ImrP, mecA, erm*(C), *mprF, sdrM*	PVL-	E
t002/ST5-MRSA-IVc	4	H3 [1] H4 [2] H21 [1]	Ap [1] Ap, Cp [2] Ap, Er [1]	*blaZ, fosB, lmrP, mecA, mprF* [1] *blaZ, fosB, ImrP, mecA, mprF, sdrM, merA/B, qacA* [1] *fosB, ImrP, mecA, mprF, sdrM, erm*(C) [1] *blaZ, fosB, ImrP, mecA, mprF, sdrM* [1]	PVL- [4]	F [2] B [1] Novel type 1 (*sep* only) [1]
t002/ST5-MRSA-IVg	2	H9 [1] H13 [1]	Ap [1] Ap, Cl [1]	*blaZ, fosB, ImrP, mecA, mprF, sdrM* [1] *fosB, fexA, ImrP, mecA, mprF, sdrM* [1]	PVL- [2]	B [1] E [1]

[a]Genotypic information was extracted from whole-genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany).

Abbreviations: *spa*, staphylococcal protein A type; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mec*; N, number; ampicillin (Ap), chloramphenicol (Cl), ciprofloxacin (Cp), erythromycin (Er), fusidic acid (Fd), rifampicin (Rf), tetracycline (Te), trimethoprim (Tp); AR, antimicrobial resistance; PVL, Pantone-Valentine leukocidin; +, positive; -, negative.

Table 4.3. Literature search to identify previously investigated PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates

Part (a): Literature search results; closest hits							
PMID	**Country**	**Year**	**DOI**	**No. of isolates in study**	**ST**	**PVL+/-**	**SCC*mec* type**
31080781	United Kingdom, Sri Lanka, Australia	2019	https://doi.org/10.3389/fcimb.2019.00123	56 (46 included in study)	ST5	PVL+	IVc
33122734	United Arab Emirates	2020	https://doi.org/10.1038/s41598-020-75565-w	14	ST5	PVL+	IVc
33939761	United States of America	2021	https://doi.org/10.1371/journal.pone.0250975	2	ST5	PVL+	IVc
33118033	Czech Republic	2021	https://doi.org/10.1093/jac/dkaa404	11	ST5	PVL+	IVc
28399994	United States of America	2017	http://dx.doi.org/10.1016/j.fm.2017.01.015	3	ST5	PVL+	Not mentioned
23916451	China	2013	http://dx.doi.org/10.1016/j.bjid.2013.02.007	6	ST5	PVL+	IV
23922169	Sweden	2013	https://doi.org/10.1007/s10096-013-1929-2	25	ST5	PVL+	Not mentioned
25838886	United States of America	2015	https://doi.org/10.1186/s13756-015-0048-5	6	ST5	PVL+	Not mentioned
29912846	Israel	2017	https://doi.org/10.1093/ofid/ofx163.1709	28	ST5	PVL+	IV
34094618	The Philippines	2021	https://doi.org/10.5365/wpsar.2020.11.1.004	2	ST5	PVL+	IV
32764618	Germany	2020	https://doi.org/10.1038/s41598-020-70112-z	4	ST5	PVL+	IV
30424799	Italy	2018	https://doi.org/10.1186/s13073-018-0593-7	10	ST5	PVL+	IV
32883933	Brazil	2020	https://doi.org/10.4103/ijmm.IJMM_20_157	2	ST5	PVL+	IV
29386909	China	2018	https://doi.org/10.2147/IDR.S153399	1	ST5	PVL+	IV
Part (b): Literature search results; partial hits (potential comparator isolates)							

Table 4.3 continued overleaf

PMID	Country	Year	DOI	No. of isolates in study	ST	PVL+/-	SCC*mec* type
34670645	Brazil	2021	https://doi.org/10.3201/eid2711.210097	167	ST5	Not mentioned	IV
23269731	USA	2013	https://doi.org/10.1128/JCM.02429-12	1	ST5	PVL+	II
23765159	Chile	2013	https://doi.org/10.1007/s10096-013-1907-8	2	ST5	PVL-	IVc, IV non-typeable
23765686	USA	2014	https://doi.org/10.1002/ppul.22815	42	ST5	PVL-	II
30322522	Sweden	2018	https://doi.org/10.1016/j.vetmic.2018.09.017	12	ST2659	PVL+	Not mentioned
29676483	China	2018	https://doi.org/10.1002/jcla.22456	29	ST5	Not clarified	III
18948410	Spain	2009	https://doi.org/10.1093/jac/dkn430	15	ST5	PVL-	IV

Abbreviations: ST, sequence type; PVL, Panton Valentine Leukocidin; +, positive; -, negative; SCC*mec* type, Staphylococcal Cassette Chromosome *mec*.

(i) Clinical isolates

Fifty-six PVL-positive ST5-MRSA-IVc isolates recovered between 2005–2021 in the Czech Republic (*N*=6), Germany (*N*=4), Kuwait (*N*=1), Norway (*N*=24), Saudi Arabia (*N*=4), Sweden (*N*=2) and UAE (*N*=15) underwent WGS at the Dublin Dental University Hospital Microbiology Research Unit (DDUH, Ireland) (Table 4.1).

(ii) WGS datasets

WGS datasets for PVL-positive ST5-MRSA-IVc isolates from Denmark (2007–2021) (*N*=66) and Germany (2011–2019) (*N*=16) were received. WGS datasets for the 46 PVL-positive ST5-MRSA-IVc Sri Lankan clone isolates previously described by McTavish *et al.,* 2019, were downloaded from ENA (accession number PRJEB27049). These patient isolates were recovered in a Sri Lankan hospital over a four-month period in 2014 (*N*=33), the UK between 2005–2015 (*N*=12) and Australia in 2015 (*N*=1) (Table 4.1).

4.2.1.4 International comparator reference isolates

The literature search and contact with international collaborators also yielded 35 international comparators (24 clinical isolates and eleven WGS sequences; Table 4.1). The twenty-four clinical ST5-MRSA-II/IVa/IVc/V comparator isolates (19 PVL-positive and five PVL-negative) were recovered in Algeria (*N*=1), the Czech Republic (*N*=2), Germany (*N*=1), Kuwait (*N*=1), Norway (*N*=12), Saudi Arabia (*N*=1), Senegal (*N*=2), Slovakia (*N*=1) and the UAE (*N*=3) between 2003 and 2021 and underwent WGS at DDUH (Table 4.1). The comparator WGS datasets were received from Denmark (seven PVL-positive and three PVL-negative; 2013–2015) and Germany (one PVL-positive; 2017).

4.2.2 NMRSARL molecular characterisation

All Irish isolates included in the current study underwent species identification and phenotypic antimicrobial susceptibility testing at the NMRSARL as described in Chapter 2, Section 2.3. The NMRSARL also performed *spa* typing on these isolates as described in Chapter 2, Section 2.3.3.

4.2.3 Genomic DNA isolation

All clinical isolates underwent genomic DNA extraction as described in Chapter 2, Section 2.4.1.

4.2.4 Whole-genome sequencing

4.2.4.1 Second-generation Illumina short-read sequencing

All clinical isolates underwent short-read WGS as described in Chapter 2, Section 2.5.1.

4.2.4.2 Third-generation ONT MinION long-read sequencing

As the cost per sample (DNA isolation, library preparation and sequencing reagents) for third generation sequencing is significantly higher than second generation sequencing, a selection of the study isolates were chosen to construct representative hybrid assemblies (combination of long-read and short-read whole-genome sequence data). Where clinical samples were available, thirty-four isolates representative of the different countries under investigation were selected for long-read sequencing (Fig. 4.3). This included Sri Lankan clone isolates (*N*=26) recovered from the Czech Republic (*N*=1), Denmark (*N*=6), Germany (*N*=2), Ireland (*N*=8), Kuwait (*N*=1), Norway (*N*=3), Saudi Arabia (*N*=2), Sweden (*N*=1) and UAE (*N*=2). Eight comparators from Algeria (*N*=1), Germany (*N*=1), Ireland (*N*=1), Norway (*N*=3), and UAE (*N*=2) also underwent long-read sequencing.

4.2.4.2.1 High molecular weight genomic DNA extraction

Isolates were re-activated from the -80°C stock onto fresh CBA plates as described in Chapter 2, Section 2.4.1. Enzymatic lysis was performed by adding 1 µl inoculating loop of the lawned culture into 200 µl of lysis buffer/lysis enhancer (Iter-Array fzmb GmbH) in a 1.5 ml tube (Eppendorf). The mixture was incubated at 37°C for 3 h in a shaking thermomixer (Eppendorf ThermoMixer® C). Following lysis, genomic DNA extraction was performed using the MagAttract High-Molecular Weight Kit (Qiagen) as per manufacturer's instructions. Briefly, 20 µl of Proteinase K, 4 µl RNase A solution and 150 µl Buffer AL (all supplied with the MagAttract Qiagen kit) were added to the 200 µl sample mixture to digest proteins in the lysate. Samples were then incubated at 15–25°C for 30 min in a shaking thermomixer (Eppendorf). Following incubation, 15 µl of MagAttract Suspension G was added to each sample, followed by 280 µl Buffer MB. Samples were then incubated again at 15–25°C for 3 min in a shaking thermomixer (Eppendorf) set to 1400 rpm. Following incubation, samples were placed onto a magnetic separation rack (NEBNext New England BioLabs, Massachusetts, USA) for 1 min to allow for bead separation and removal of supernatant. DNA isolation was performed according to manufacturer's instructions using 700 µl Buffer MW1 and Buffer PE (supplied with the MagAttract Qiagen kit). Extracted DNA was eluted in 100 µl of sterile

molecular-grade water in fresh Eppendorf tubes. Samples were stored at 4°C (short-term) or at -20°C (long-term). The concentration/purity of extracted DNA was measured as described in Chapter 2, Section 2.4.2.

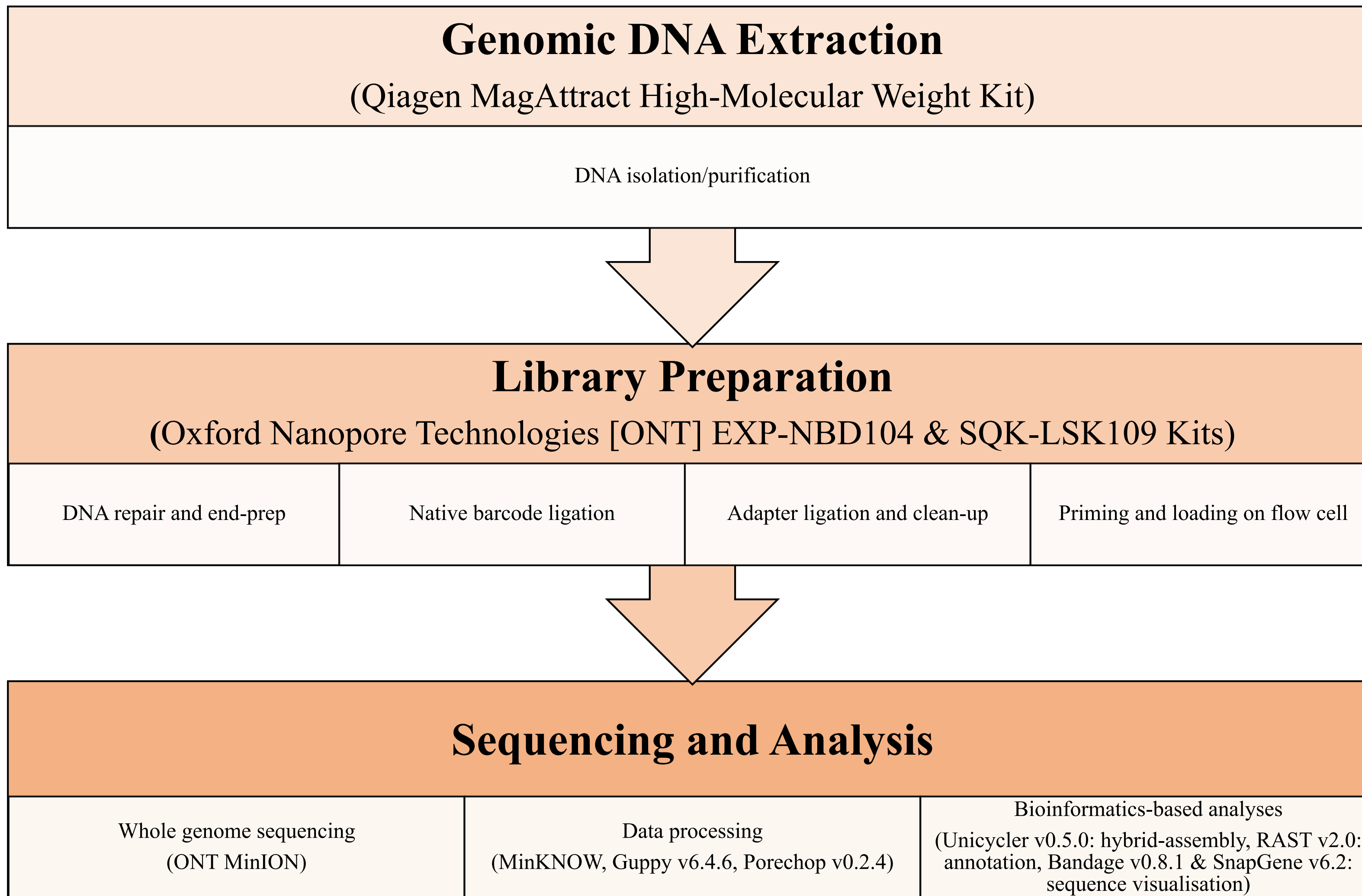

Figure 4.3. Simplified workflow of the long-read whole genome sequencing process employed for the present study.

4.2.4.2.2 Library preparation

Library preparation was performed with the Native Barcoding Expansion 1–12 (EXP-NBD104) kit and the Ligation Sequencing (SQK-LSK109) kit (ONT, Oxford, UK). Sequencing was performed on the MinION platform using a R9.4.1 SpotON flow cell with the MinKNOW software v20.10 (ONT) as per manufacturer's instructions.

(i) DNA repair and end-prep

For each isolate included in the library, 1.5 µg of HMW gDNA was adjusted to a volume of 48 µl in sterile molecular-grade water. The 48 µl gDNA sample was added to a master mix containing 3.5 µl NEBNext FFPE DNA Repair Buffer, 3.5 µl Ultra II End-prep reaction buffer, 3 µl Ultra II End-prep enzyme mix and 2 µl NEBNext FFPE DNA Repair Mix (all supplied by New England BioLabs). The master mix was incubated in a PCR thermal cycler (Biometra TOne) at 20°C for 10 min and 65°C for 10 min. Following incubation, 60 µl of resuspended Agencourt AMPure XP beads (Beckman Coulter, California, USA) was added to the end-prep reaction mix. The samples were incubated on a Hula mixer (Fisher Scientific™) for 25 min at room temperature. Following incubation, samples were placed onto a magnetic separation rack (New England BioLabs) for 1 min to pellet beads and pipette off the supernatant. While on the magnet, the beads were washed twice with 200 µl of freshly prepared 70% ethanol without disrupting the pellet. After pipetting off any residual ethanol, the beads were left to dry for ~30 s, before removal from the rack and resuspension in 28 µl of sterile water. The samples were then incubated at room temperature for 20 min, and placed back onto the magnetic rack. When clear and colourless, 26 µl of the eluate was removed and retained in fresh 1.5 ml Eppendorf tubes. The repaired and end-prepped DNA samples were quantified using a Qubit Fluorometer 3.0 (Invitrogen/Fisher Scientific™) with a recovery aim >700 ng.

(ii) Native barcode ligation

For native barcode ligation, 800 ng of each end-prepped sample to be barcoded was prepared in 22.5 µl of sterile water. The following reagents were added to the DNA samples in the order given below, mixing by flicking the tube between each sequential addition: 2.5 µl Native Barcode (ONT) and 25 µl Blunt/TA Ligase Master Mix (New England BioLabs). The mix was incubated at room temperature for 20 min. Following incubation, 50 µl of resuspended AMPure XP beads was added to the reaction and the

samples were incubate on the Hula mixer for 25 min at room temperature. Samples were placed on the magnetic rack to pellet beads, followed by washing of beads and removal of eluate as previously described in the 'DNA repair and end-prep' section. Equimolar amounts of each barcoded sample was pooled into a fresh Eppendorf tube. The pool was quantified on the Qubit Fluorometer 3.0 with a recovery aim >700 ng. The >700 ng pooled sample was diluted to a volume of 65 µl in sterile water.

(iii) Adapter ligation and clean-up

To perform adapter ligation, the following reagents were added to the 65 µl pooled/barcoded DNA samples in the order given below, mixing by flicking the tube between each sequential addition: 5 µl Adapter Mix II (AMII) (ONT), 20 µl NEBNext Quick Ligation Reaction Buffer (5X) and 10 µl Quick T4 DNA Ligase (both supplied by New England BioLabs). The mix was incubated at room temperature for 10 min. Following incubation, 50 µl of resuspended AMPure XP beads were added to the reaction and the samples were incubate on the Hula mixer for 25 min at room temperature. Following incubation, samples were placed onto a magnetic separation rack (New England BioLabs) for 1 min to pellet beads and pipette off the supernatant. The beads were then washed and resuspended with 250 µl Long Fragment Buffer (LFB) (ONT) twice. This clean-up step removed any unprepared DNA from the library preparation. The beads were placed back onto the magnetic rack to pellet and residual LFB was pipetted off. The beads were left to dry for ~30 s, before removal from the magnetic rack and resuspension in 17 µl of Elution Buffer (EB) (ONT). The samples were then incubated at 37°C for 20 min in a static incubator (Gallenkamp), before being placed back onto the magnetic rack. When clear and colourless, 15 µl of the eluate was removed and retained in fresh 1.5 ml Eppendorf tubes. The adapter ligated/barcoded pooled sample was quantified using the Qubit Fluorometer 3.0 with a recovery aim of ~430 ng.

(iv) Priming and loading on flow cell

For priming and loading of the SpotON flow cell, the visual guide provided by ONT was followed (Available at: https://youtu.be/Pt-iaemrM88). Briefly, 5–50 fmol of the final prepared library was loaded onto the flow cell via the SpotON sample port in a dropwise fashion. The ports were closed gently along with the MinION device and the sequencing run was commenced using the MinKNOW software v20.10 (ONT). MinKNOW allowed for real-time viewing of reads processed and sequencing run metrics.

(v) Post-sequencing processing

On completion of the run, the generated raw-reads (FAST5 files) were processed using command-line bioinformatic tools. The reads underwent base-calling using Guppy v6.4.6 (ONT; https://github.com/nanoporetech/rerio). Adapter trimming and demultiplexing of the sequence reads was performed using Porechop v0.2.4 (https://github.com/rrwick/Porechop). The output generated were separate FASTQ files for each sequenced isolate.

4.2.4.3 Hybrid assembly

Isolates selected for hybrid assembly underwent genome scaffolding using paired-end short-read MiSeq sequences (Illumina) and long-read MinION sequences (ONT) using the Unicycler v0.5.0 pipeline (https://github.com/rrwick/Unicycler). Short-read assemblies and hybrid-assembled genomes were annotated using the web-based RAST v2.0 server (https://rast.nmpdr.org). Visualisation of assembled genomes was performed using Bandage v0.8.1 (https://rrwick.github.io/Bandage/) and SnapGene v6.2 (GSL Biotech LLC; https://www.snapgene.com).

4.2.5 Bioinformatic analyses

All study isolates underwent *de novo* assembly, *spa* type confirmation, SCC*mec* subtyping, MLST, *in silico* antimicrobial resistance profiling and virulence gene identification as described in Chapter 2, Section 2.6.

4.2.5.1 Molecular characterisation

In silico DNA microarray profiling using probes of the *S. aureus* Genotyping Kit 2.0 (Abbott) was performed as previously described (Monecke *et al.,* 2016). Briefly, *in silico* oligonucleotide probes corresponding to ~170 *S. aureus* target sequences were used to assign CCs, STs and identify virulence and pathogenicity-associated genes. Probe sequences were mapped onto assembled genomes of the study isolates to predict DNA array hybridization patterns and these patterns were compared with *in vitro* array results (Monecke *et al.,* 2016).

Additional investigations into alleles of interest were performed using the European Bioinformatics Institute (EBI) multiple sequence alignment tool, Clustal Omega

(https://www.ebi.ac.uk/Tools/msa/clustalo/) and the BLAST search engine (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.2.5.2 Maximum likelihood analysis

A maximum likelihood phylogenetic analysis based on core-genome SNPs was performed for (i) 266 Irish and international isolates (214 Sri Lankan clone isolates and 52 comparators) under investigation (ii) 47 Irish isolates (40 ST5-MRSA-IVc/V and seven comparators) under investigation. Firstly, core-genome alignment and SNP-based variant calling was performed on all study isolates. The isolates were mapped against a study-specific reference genome (isolate 141087; oldest Sri Lankan clone isolate investigated [2005])/(isolate M150014; oldest Irish ST5-MRSA study isolate [2015]), using Snippy v4.6.0 (https://github.com/tseemann/snippy). Recombinant SNPs were removed using Gubbins v3.2.1 (https://github.com/nickjcroucher/gubbins) and a pairwise cgSNP distance matrix was generated using snp-dists v3 (https://github.com/tseemann/snp-dists). A cgSNP-based maximum-likelihood tree (MLT) was constructed through IQ-TREE v2.2.0 (http://www.iqtree.org) using recommended IQ-TREE guidelines. A minimum number of bootstrap replicates (1000) was used and the general time reversible (GTR) + ascertainment bias correction (ASC) substitution model was applied to the SNP data, as recommended by IQ-TREE guidelines. The phylogenetic tree was visualized and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

4.2.5.3 Minimum spanning tree

A cgMLST-based MST was constructed for 266 Irish and international isolates (214 Sri Lankan clone isolates and 52 comparators) under investigation as described in Chapter 2, Section 2.6.5.1. A cgMLST-based and wgMLST-based MST was constructed for 47 Irish isolates (40 ST5-MRSA-IVc/V and seven comparators) under investigation as described in Chapter 2, Section 2.6.5.1.

4.3 Results

4.3.1 The CC5/ST5-MRSA-IVc Sri Lankan clone

To characterise the PVL-positive ST5-MRSA-IVc Sri Lankan clone in detail based on its molecular epidemiology and phylogenetic diversity, 266 MRSA isolates recovered between 2003 and 2022 were investigated. This included 214 PVL-positive CC5/ST5-MRSA-IVc isolates from 12 countries similar to and including 46 'Sri Lankan clone' isolates and 29 PVL-positive and 23 PVL-negative ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates (Table 4.1). The PVL-positive ST5-MRSA-IVc isolates belonged to eight closely related *spa* types (t002, t010, t306, t045, t062, t1062, t535 and t579), with t002 predominating (186/214; 86.9%) (Table 4.4). Six closely related *spa* types were identified among the comparator isolates (t002, t105, t311, t442, t450 and t3089), half of which were also t002 (26/52; 50%) (Table 4.4).

Where detailed clinical metadata were available (168/214; 78.5%), just over half of the PVL-positive ST5-MRSA-IVc isolates were CA-MRSA, defined as isolates recovered from patients with no hospitalisation history within the six months prior to isolate recovery (85/168; 50.6%), while the remainder (50/168; 29.8%) were HA-MRSA or were from hospitalized patients (33/168; 19.6%). Most isolates were from infection sites (142/214; 66.3%), with the remainder from carriage (50/214; 23.4%) or unknown sites (22/214; 10.3%) (Table 4.5). The majority of infection isolates were from SSTIs (83/142, 58.5%), other infection types (9/142, 6.3%) or were unknown (50/142, 35.2%). Information available for the comparator isolates (34/52; 65.4%) showed that these isolates were predominantly HA-MRSA isolates (22/34; 64.7%) followed by CA-MRSA (12/34; 35.3%). The comparator samples were recovered from carriage sites (18/52; 34.6%), infection sites (24/52; 46.2%) including SSTI (N=21), other (N=3) or unknown sites (10/52; 19.2%) (Table 4.5).

Table 4.4. *spa* type and repeat profiles of 214 CC5/ST5-MRSA-IVc Sri Lankan clone isolates and 52 CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates investigated [a]*spa* types were confirmed using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) *spa*-typing task template

***spa* type[a] [*N*]**	***spa* type repeat succession**	**No. of repeat differences to t002**
Sri Lankan clone [214]		
t002 [186]	26-23-17-34-17-20-17-12-17-16	0
t010 [2]	26-17-34-17-20-17-12-17-16	1
t306 [1]	26-23-17-34-17-20-17-12-17-17-16	1
t045 [3]	26-17-20-17-12-17-16	3
t062 [6]	26-23-17-12-17-16	4
t1062 [2]	26-23-17-34-17-02-17-12-17-16	1
t535 [2]	26-17-16	7
t579 [1]	26-23-17-17-20-17-12-17-16	1
ND [11]		
Comparators [52]		
t002 [26]	26-23-17-34-17-20-17-12-17-16	0
t105 [5]	26-23-17-34-17-20-17-17-16	1
t311 [18]	26-23-17-34-20-17-12-17-16	1
t442 [1]	35-17-34-17-20-17-12-17-16	2
t450 [1]	26-23-17-34-16	5
t3089 [1]	26-23-17-34-34-20-17-17-16	2

Abbreviations: *spa*, staphylococcal protein A; no., number; ND, not determined.

Table 4.5. Details of clades (I-III) and outgroup observed in the cgSNP-based MLT of 214 CC5/ST5-MRSA-IVc Sri Lankan clone isolates and 52 CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates investigated

Clade	No. of isolates	Isolate group	Sources [*N*]	Infection/Carriage	Recovery dates	*spa* types[a] [*N*]	PVL(+/-)
I	209	Sri Lankan clone	Australia [1], Czech Republic [6], Denmark [66], Germany [20], Ireland [29], Kuwait [1], Norway [24], Saudi Arabia [3], Sri Lanka [33], Sweden [2], UAE [12], UK [12]	Infection [138] Carriage [50] Unknown [21]	2005–2022	t002 [183] t010 [2] t045 [2] t062 [6] t1062 [2] t535 [2] t579 [1] ND [11]	PVL+ [209]
II	7	Comparators	Denmark [2], Ireland [2], Norway [2], UAE [1]	Infection [4] Carriage [2] Unknown [1]	2009–2018	t002 [7]	PVL+ [7]
III	5	Sri Lankan clone	Ireland [1], Saudi Arabia [1], UAE [3]	Infection [4] Unknown [1]	2010–2019	t002 [3] t045 [1] t306 [1]	PVL+ [5]
Neighbouring Clade III	1	Comparator	Kuwait	Unknown	2013	t002	PVL+
Outgroup	44	Comparators	Algeria [1], Czech Republic [2], Denmark [8], Germany [2], Ireland [15], Norway [10], Saudi Arabia [1], Senegal [2], Slovakia [1], UAE [2]	Infection [20] Carriage [16] Unknown [8]	2003–2021	t002 [18] t105 [5] t3089 [1] t311 [18] t442 [1] t450 [1]	PVL+ [21] PVL- [23]

[a]*spa* types assigned using Ridom Seqsphere+ v4.1 (Ridom GmbH, Münster, Germany).

Abbreviations: N, number; *spa*, staphylococcal protein A type; PVL, Panton-Valentine Leukocidin.

Epidemiological information available for some of the PVL-positive ST5-MRSA-IVc isolates from Denmark (*N*=9), Ireland (*N*=2) and the UAE (*N*=1) revealed that these isolates were from patients with international links. The Irish isolates were recovered from patients with a history of travel to Sri Lanka and Turkey. Within the Danish subset, one patient had been hospitalized in Vietnam, four were from Sri Lanka and four had travelled to Sri Lanka. The UAE isolate was from a patient from Bangladesh. Epidemiological data available for the comparator isolates revealed that isolates from Norway (*N*=3) were from a patient from Afghanistan, a patient who had travelled to Vietnam, and another with a history of hospitalisation in Brazil. Additionally, a Danish comparator isolate was recovered from a patient with a Sri Lankan name.

4.3.2 Phylogenetic analysis of Sri Lankan clone isolates

To investigate the population structure of the Irish PVL-positive CC5/ST5-MRSA-IVc isolates relative to international strains, all isolates and comparators were subjected to WGS-based phylogenetic analyses. The construction of SNP-based MLT and cgMLST-based MST trees yielded comparable findings regarding isolate relatedness and clustering (Figs. 4.4 and 4.5).

4.3.2.1 Core-genome SNP analysis

CgSNP analysis based on 12,245 SNPs showed that all 214 PVL-positive CC5/ST5-MRSA-IVc isolates exhibited a pairwise SNP-distance median of 107 (average: 116; range: 0–410). The SNP-based MLT grouped the vast majority of isolates (209/214, 97.7%) including all 46 'Sri Lankan clone' isolates into one clade, termed Clade I (Fig. 4.4). Clade I isolates had a median of 106 (average: 110; range: 0–287) SNPs between isolates. The remaining five PVL-positive ST5-MRSA-IVc grouped into Clade III exhibiting a median of 86 (average: 92; range: 69–127) SNPs (Fig. 4.4). The isolates within Clade III included a *spa* type t002 isolate recovered from Saudi Arabia in 2010, a t002 isolate recovered from Ireland in 2013 and three t002/t306/t045 isolates recovered from UAE between 2018–2019 (Table 4.5). Clade III differed from Clade I by a median of 232 (average: 237; range: 159–410) SNPs. Most comparator isolates (44/52) formed an outgroup at the base of the MLT. A single PVL-positive CC5/ST5-MRSA-IVa comparator (isolate Z4294; *spa* type t002 recovered in Kuwait in 2013) branched out next to Clade III (thick black branch in Fig. 4.4).

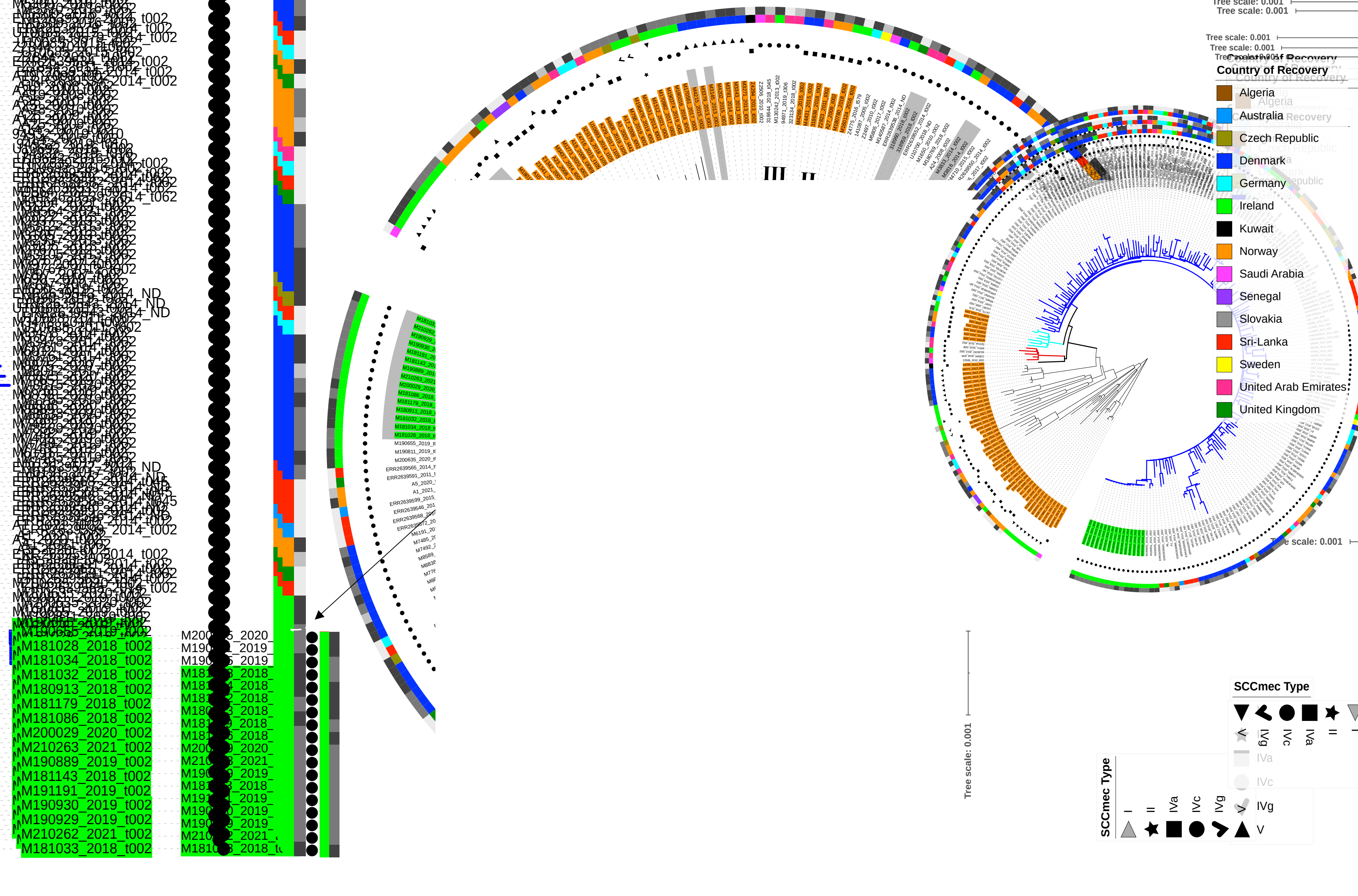
M181028_2018_t002
M181034_2018_t002
M181032_2018_t002
M180913_2018_t002
M181179_2018_t002
M181086_2018_t002
M200029_2020_t002
M210263_2021_t002
M190889_2019_t002
M181143_2018_t002
M191191_2019_t002
M190930_2019_t002
M190929_2019_t002
M210262_2021_t002
M181033_2018_t002
Tree scale: 0.001
Country of Recovery
Algeria
Australia
Czech Republic
Denmark
Germany
Ireland
Kuwait
Norway
Saudi Arabia
Senegal
Slovakia
Sri-Lanka
Sweden
United Arab Emirates
United Kingdom
SCCmec Type
I
II
IVa
IVc
IVg
V

Figure 4.4. Maximum likelihood tree (MLT) based on phylogenetic analysis of 12,245 core-genome single nucleotide polymorphisms (cgSNPs) for 214 Panton-Valentine leukocidin (PVL)-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates and 52 additional PVL-positive (N=29) and PVL-negative (*N*=23) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Sri Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia and the Middle East between 2005–2022. Separate node colours/shapes represent country of recovery, sample types and SCCmec types as indicated in the legend. Blue branches represent PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates (*N*=209/214) forming Clade I. Green branches represent PVL-positive CC5/ST5-MRSA-IVa comparator isolates (*N*=7/52) forming Clade II. Red branches represent the remaining PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates (*N*=5/214) forming Clade III. The thick black branch represents a PVL-positive CC5/ST5-MRSA-IVa comparator isolate (*N*=1) branching out next to Clade III. The thin black branches represent the comparator outgroup isolates (*N*=44) which separate away from Clades I–III. Labels for the 52 comparator isolates are highlighted in orange. Isolate names, year of recovery and *spa* types are all indicated in the branch labelling. Country-specific isolate pairs or clusters containing closely related isolates that differed by ≤10 cgSNPs are shaded in grey. The divergent subgroup of 15 Irish isolates (lacking the bbp gene) within the large Sri Lankan clade (Clade I) is indicated by an asterisk and the isolate names are highlighted in green. The epidemiological and genotypic information for each isolate investigated is provided in Table 4.1. The cgSNP-based MLT was constructed using IQ-TREE v2.2.0 (http://www.iqtree.org) and the phylogenetic tree was visualised and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

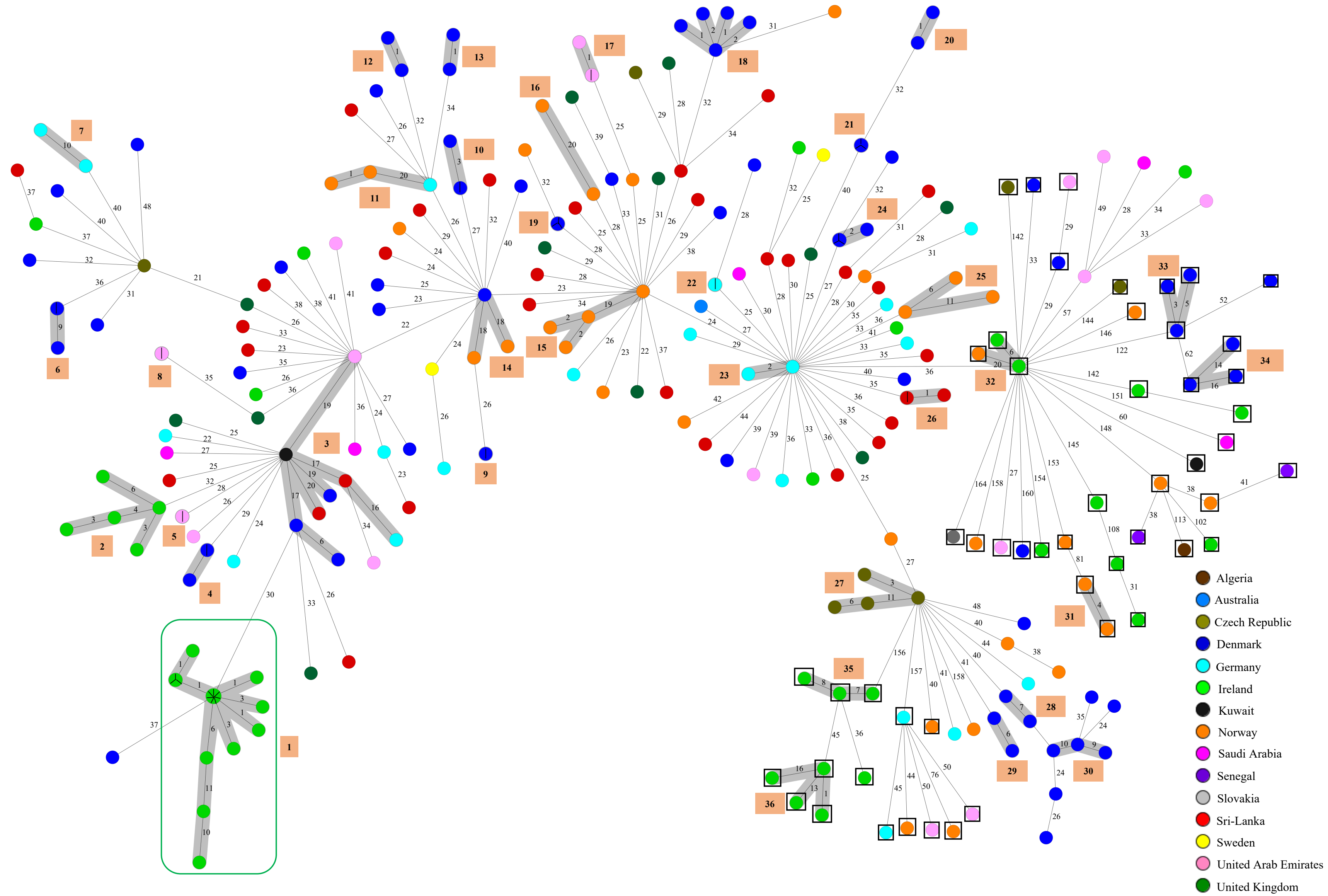
Algeria
Australia
Czech Republic
Denmark
Germany
Ireland
Kuwait
Norway
Saudi Arabia
Senegal
Slovakia
Sri-Lanka
Sweden
United Arab Emirates
United Kingdom

Figure 4.5. Minimum spanning tree (MST) based on core genome multi-locus sequence type (cgMLST) analysis of 1,861 target genes for 214 Panton-Valentine leukocidin (PVL)-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates and 52 additional PVL-positive (*N*=29) and PVL-negative (*N*=23) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Sri Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia, and the Middle East between 2005–2022. Separate node colours represent country of isolation as indicated in the legend. Partitions within nodes represent the presence of ≥2 isolates per node. Comparator isolates are indicated in the MST by black squares. Closely related clusters of isolates (≤20 allelic differences to the closest neighbouring isolate within the RIG) are outlined within grey shadowing. Branch numbers indicate the number of allelic differences between neighbouring isolates. Node numbers indicate the 36 related isolate groups (RIGs) in the population (Table 4.5). The subgroup of closely related Irish isolates consisting of 15 isolates with a distinct genotypic profile to other Sri Lankan clone isolates is outlined in green. The genotypic and epidemiological information for each isolate is provided in Table 4.1. The cgMLST-based MST was constructed using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany).

The remaining seven comparators (all PVL-positive ST5/t002-MRSA-IVa) grouped into Clade II, forming the closest neighbour to Clade I (Fig. 4.4). The comparator isolates within Clade II included two isolates recovered from Denmark in 2015, two from Ireland between 2016–2018, two from Norway between 2009–2011 and one isolate recovered from UAE in 2018 (Table 4.5). Clade II differed from Clades I and III by a median of 176 (average: 178; range: 123–331) and 230 (average: 234; range: 207–277) SNPs, respectively. In general, the PVL-positive ST5-MRSA-IVc isolates did not group according to their country of origin or year of recovery; however, 24 small country-specific clusters of closely related isolates that differed by ≤10 cgSNPs were evident (shaded in grey in Fig. 4.4).

4.3.2.2 Core-genome MLST analysis

The PVL-positive ST5-MRSA-IVc isolates and comparators were also investigated by cgMLST analysis. As cgSNP analysis revealed low genotypic diversity among these isolates recovered over 17 years, the previously recommended threshold of ≤24 cgMLST allelic differences for defining closely related isolates (Schürch *et al.,* 2018) was lowered to ≤20. The 214 PVL-positive ST5-MRSA-IVc isolates exhibited a median of 55 allelic differences from one another (average: 59; range: 0–200). The 209 Clade I and five Clade III isolates exhibited a median of 54 (average: 57; range: 0–153) and 42 (average: 43; range: 28–56) allelic differences, respectively. Clade III isolates differed from Clade I by a median of 116 (average: 117; range: 70–200) allelic differences. Clade II comparator isolates differed from Sri Lankan clone Clade I and Clade III by a median of 87 (average: 86; range: 36–174) and 106 (average: 93; range: 28–134) allelic differences, respectively.

Although the findings of the phylogenetic analysis confirmed limited diversity between isolates within Clade I (ST5-MRSA-IVc), Clade II (ST5-MRSA-IVa) and Clade III (ST5-MRSA-IVc), McTavish *et al*., 2019, described the 'Sri Lankan clone' as a PVL-positive ST5-MRSA lineage harbouring a type IVc SCC*mec* element. In accordance with this definition, only the 214 CC5/ST5-MRSA-IVc isolates within Clades I and III are hereafter referred to as 'Sri Lankan clone'. The tree topology observed in the cgSNP analysis where Clade II ST5-MRSA-IVa isolates grouped between Sri Lankan clone Clade I and III suggests that this PVL-positive ST5-MRSA-IVa lineage originally emerged from a common progenitor as the PVL-positive ST5-MRSA-IVc Sri Lankan clone and acquired a different SCC*mec* subtype.

Thirty-six related isolate groups (RIGs) comprising 123/266 study isolates were evident in the cgMLST-based MST (Fig. 4.5 and Table 4.6). Isolates within each RIG exhibited ≤20 allelic differences to the closest neighbouring isolate in the RIG. Most RIGs included Sri Lankan clone isolates only (30/36 RIGs) and the remaining six (RIGs 31–36) included comparator isolates only (Fig. 4.5). There were 32 country-specific RIGs (27 Sri Lankan clone isolates only (RIGs 1–2, 4–10, 12–13, 15–30) and five comparator isolates only (RIGs 31, 33–36)) as follows: Denmark (*N*=16), Norway (*N*=4), Ireland (*N*=4), Germany (*N*=3), UAE (*N*=3), Sri Lanka (*N*=1) and Czech Republic (*N*=1). The remaining four RIGs comprised isolates from two or more countries (Fig. 4.5 and Table 4.6). RIG-3 comprised eight Sri Lankan clone isolates from Denmark (*N*=3), Sri Lanka (*N*=2), Germany (*N*=1), Kuwait (*N*=1) and the UAE (*N*=1), with an allelic difference range of 6–20 between neighbouring isolates in the RIG and a range of 6–36 allelic differences for the entire RIG (Fig. 4.5). Sri Lankan clone isolates from Norway (*N*=2) formed two separate RIGs (RIG-11 and RIG-14) with Sri Lankan clone isolates from Germany (*N*=1) and Denmark (*N*=1) with allelic difference ranges of 1–21 and 18–20, respectively. One comparator isolate from Norway formed a third RIG (RIG-32) with two comparator isolates from Ireland. The close relatedness between these isolates from disparate geographic regions indicated international transmission of the Sri Lankan clone.

Table 4.6. Genotypic details for 36 related isolate groups (RIGs); of the 266 (214 CC5/ST5-MRSA-IVc Sri Lankan clone isolates, 29 PVL-positive and 23 PVL-negative CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator strains) study isolates, 123 (105 Sri Lankan clone isolates and 18 comparator isolates) formed RIGs

RIG[a]	Isolate [*N*]	Country [*N*]	Years of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[b] [*N*]	PVL(+/-)	IEC type [*N*]
1	18	Ireland	2018–2021	t002-ST5-IVc	*blaZ* [17], *fosB* [18], *lmrP* [18], *mprF* [18], *sdrM* [18]	+	G
2	5	Ireland	2020–2022	t002-ST5-IVc	*blaZ* [5], *fosB* [5], *lmrP* [5], *mprF [*5], *sdrM* [5], *erm*(C) [4]	+	G
3	8	Denmark [3] Germany [1] Kuwait [1] Sri Lanka [2] United Arab Emirates [1]	2013–2019	t002-ST5-IVc	*blaZ* [8], *fosB* [8], *lmrP* [8], *mprF* [8], *sdrM* [8], *erm*(C) [6], *tetK* [1]	+	G
4	3	Denmark	2018–2019	t002-ST5-IVc [2] t002-ST5-IVa [1]	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [2]	+	G
5	2	United Arab Emirates	2017	t002-ST5-IVc	*blaZ* [2], *erm*(C) [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	G
6	3	Denmark	2017–2020	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [2]	+	G
7	2	Germany	2019	t535-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	G
8	2	United Arab Emirates	2018	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	G
9	2	Denmark	2014	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	F
10	3	Denmark	2015–2016	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G
11	3	Norway [2] Germany [1]	2011–2013	t002-ST5-IVc [2] ND-ST5-IVc [1]	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [1], *tet*(K) [1]	+	G

Table 4.6 continued overleaf

RIG[a]	Isolate [*N*]	Country [*N*]	Years of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[b] [*N*]	PVL(+/-)	IEC type [*N*]
12	2	Denmark	2012–2015	t002-ST5-IVc	*blaZ* [2], *fosB* [1], *lmrP* [1], *mprF* [2], *sdrM* [2], *erm*(C) [2]	+	G
13	2	Denmark	2015–2016	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2], *erm*(C) [2]	+	G
14	3	Norway [2] Denmark [1]	2008–2010	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [2], *tet*(K) [1]	+	G
15	4	Norway	2007–2010	t002-ST5-IVc	*blaZ* [4], *fosB* [4], *lmrP* [4], *mprF* [4], *sdrM* [4]	+	G
16	2	Norway	2007–2008	t002-ST5-IVc	*blaZ* [1], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2], *erm*(C) [1]	+	G
17	3	United Arab Emirates	2019	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G
18	5	Denmark	2016–2017	t002-ST5-IVc	*blaZ* [5], *fosB* [5], *lmrP* [5], *mprF* [5], *sdrM* [5], *erm*(C) [5]	+	G
19	3	Denmark	2016	t002-ST5-IVc	*fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [2], *mupA* [3], *qacA* [3]	+	F
20	2	Denmark	2019	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	G
21	3	Denmark	2015	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G
22	2	Germany	2012	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	Novel Type 2 (*sak, scn, sea, sep*)
23	2	Germany	2013–2014	t002-ST5-IVc	*blaZ* [1], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [1], *erm*(C) [2]	+	G
24	4	Denmark	2012–2013	t002-ST5-IVc	*blaZ* [4], *fosB* [4], *lmrP* [4], *mprF* [4], *sdrM* [4], *erm*(C) [3], *tet*(K) [2]	+	G
25	3	Norway	2020	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [3]	+	G

Table 4.6 continued overleaf

RIG[a]	Isolate [*N*]	Country [*N*]	Years of isolation	*spa*-ST-SCC*mec* [*N*]	AR genes[b] [*N*]	PVL(+/-)	IEC type [*N*]
26	3	Sri Lanka	2014	t002-ST5-IVc t010-ST5-IVc t045-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G
27	4	Czech Republic	2018–2021	t002-ST5-IVc	*blaZ* [4], *fosB* [4], *lmrP* [4], *sdrM* [4], *mprF* [4]	+	G [3] Novel Type 3 (*sak, sep*)
28	2	Denmark	2018–2019	t002-ST5-IVc t002-ST5-IVa	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2]	+	G
29	2	Denmark	2018–2019	t002-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2], *erm*(C) [1]	+	G
30	3	Denmark	2017–2019	t002-ST5-IVc	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3]	+	G
*31	2	Norway	2005–2008	t311-ST5-IVc	*blaZ* [2], *fosB* [2], *lmrP* [2], *mprF* [2], *sdrM* [2], *aphA3* [2], *sat* [2]	+	A
*32	3	Ireland [2] Norway [1]	2009–2018	t002-ST5-IVa [2] t002-ST5-IVa [1]	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *erm*(C) [3]	+	G
*33	3	Denmark	2013–2015	t002-ST5-V	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *aac-aphD* [2]	+ [2] - [1]	G
*34	3	Denmark	2013–2014	t002-ST5-V	*blaZ* [3], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *aacA-aphD* [3]	+ [2] - [1]	G
*35	3	Ireland	2015–2019	t311-ST5-V	*blaZ* [2], *fosB* [3], *lmrP* [3], *mprF* [3], *sdrM* [3], *fusC* [3], *erm*(C) [2]	-	E
*36	4	Ireland	2017–2019	t311-ST5-V	*blaZ* [4], *fosB* [4], *lmrP* [4], *mprF* [4], *sdrM* [4], *fusC* [4], *erm*(C) [3]	-	E

[a]RIGs were defined as groups of isolates exhibiting ≤20 cgMLST allelic differences to their closest neighbouring isolate within the RIG. RIGs identified following core-genome multi-locus sequence type minimum spanning tree (cgMLST MST) analysis using SeqSphere+.

[b]Genotypic information extracted from whole genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) and *S. aureus* Genotyping Kit 2.0 (Abbott [Alere]) microarray profiling.

*Asterisk indicates RIGs containing only comparator isolates.

Abbreviations: RIG, Related isolate group; n, number; ND, not determined – isolates not available and *spa* types could not be determined using *in silico* techniques on the available genomic sequence data; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; PVL, Pantone-Valentine leukocidin; +, positive; -, negative; IEC, immune evasion cluster; s*ep*, staphylococcal enterotoxin p gene.

4.3.2.3 Analysis of sub-clades

Potential sub-clades (RIGs 1–36) observed on the cgSNP-based MLT and cgMLST-based MST phylogenetic trees were further investigated using *in silico* DNA microarray profiling and WGS data to identify possible RIG/country-specific characteristics. A genotypic difference was observed between the overall Sri Lankan clone population and 15/18 Irish Sri Lankan clone isolates in RIG-1 (Table 4.6). These 15 isolates (shaded in green in Figs. 4.4 and 4.5) formed a distinct Irish sub-clade within the cgSNP-based MLT (Fig. 4.4) and lacked the *bbp* gene (also known as *sdrE*) encoding a surface-associated, bone sialoprotein-binding protein. The absence of *bbp* in these 15 isolates was confirmed by analysing hybrid assembled genomes (Fig. 4.6). The absence of the *bbp* gene in only this Irish sub-clade suggested that local divergence of the Sri Lankan clone could potentially have occurred in Ireland.

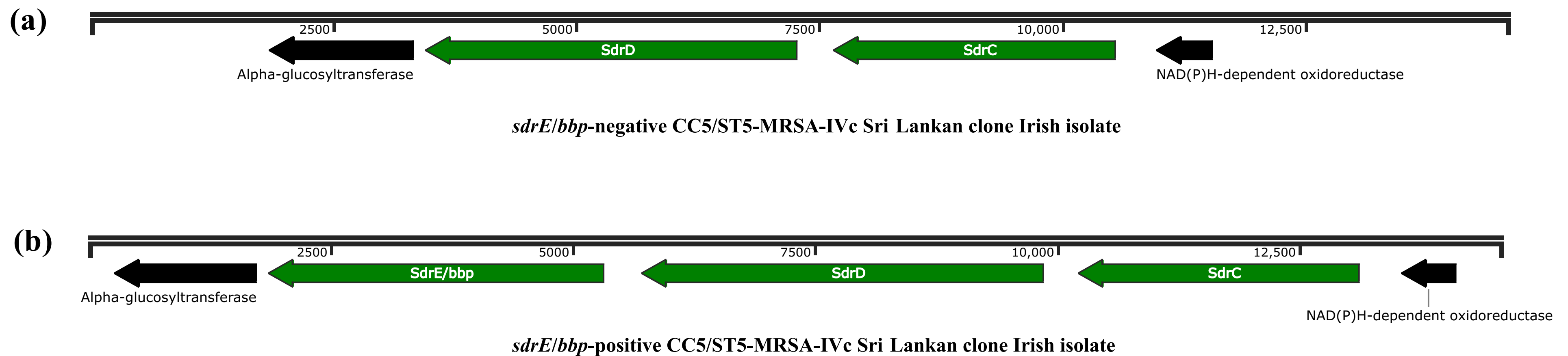

Figure 4.6. Comparative structural organisation of the serine-aspartate repeat protein-encoding (*sdr)* locus of **(a)** the *sdrE/bbp*-negative PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone Irish isolate M181179 and **(b)** the *sdrE/bbp*-positive PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone Irish isolate M180912. The tandemly arranged *sdr* genes *sdrC* (~2904 bp), *sdrD* (~4140 bp) and *sdrE* (~3462 bp) encode Sdr surface proteins (members of the Microbial Surface Components Recognising Adhesive Matrix Molecules [MSCRAMM] family). A divergent subgroup of 15 PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone Irish isolates recovered between 2018–2021 lacked this *sdrE*/*bbp* gene. Isolate sequences underwent hybrid assembly following long-read and short-read sequencing. The hybrid assembled genomes were annotated using RAST v2.0 (https://rast.nmpdr.org) and visualised using SnapGene v6.0.6 (https://www.snapgene.com).

4.3.3 Genotypic profiling of the Sri Lankan clone

4.3.3.1 Strain assignment

DNA microarray profiling, SCC*mec*Finder and Ridom Seqsphere+ template tools for detection of antimicrobial-resistance and virulence-associated genes revealed that most genes in Sri Lankan clone isolates (*N*=214) were homogenously distributed. Microarray analysis grouped Sri Lankan clone isolates into two categories including 'CC5-MRSA-IVc (*sed*/*sej*/*ser*+)' (200/214; 93.5%) and 'CC5-MRSA-IVc (*sed*/*sej*/*ser*-)' (14/214; 6.5%). The two groups differed by the presence/absence of the *sed*/*sej*/*ser* enterotoxin genes, which were located on a plasmid of approximately 27 kb (Fig. 4.2). Of the five Sri Lankan clone Clade III isolates (Fig. 4.4), four isolates were "CC5-MRSA-IVc (*sed*/*sej*/*ser*-)".

4.3.3.2 Antimicrobial resistance genes

The *β-lactamase encoding* gene *blaZ* was harboured by the majority of the Sri Lankan clone isolates (202/214; 94.4%). The *fosB* gene encoding fosfomycin resistance and the *sdrM* gene encoding a putative multi-drug efflux pump were also detected in the majority of Sri Lankan clone isolates (213/214; 99.5%). The *sdrM* gene typically encodes resistance to norfloxacin, acriflavine and ethidium bromide antimicrobial agents. The *lmrP* gene encoding a multidrug transporter mediating resistance against macrolides, lincosamides, streptogramins and tetracycline was also detected in most isolates (212/214; 99.1%). Additional antimicrobial resistance genes variably detected among the Sri Lankan clone isolates included those conferring resistance to erythromycin [*erm*(C)] (88/214; 41.1%) and tetracycline [*tet*(K)] (13/214; 6.1%) (Table 4.1).

4.3.3.3 Immune evasion cluster types

IEC-type G was predominant amongst Sri Lankan clone isolates (196/214; 91.6%) (Fig. 1.4). The remaining isolates harboured IEC-type F (7/214; 3.3%), IEC-type E (2/214; 0.9%), IEC-type D (1/214; 0.5%) or were non-typeable IEC variants harbouring *sep* only (2/214; 0.9%), *sak* and *sep* (1/214; 0.5%) or *sak*, *scn* and *sea-sep* (4/214; 1.9%). One Sri Lankan clone isolate recovered from Ireland in 2013 (M130242) lacked lysogenic β-haemolysin converting bacteriophages, carried no IEC genes and harboured an intact, untruncated *hlb* gene.

4.3.4 Comparative investigation of similar PVL-positive and PVL-negative ST5-MRSA in Irish hospitals

Within the context of the previously confirmed ST5-associated outbreak in an Irish hospital (see Chapter 3, Section 3.3.2.2), together with the increase in ST5-MRSA isolates submitted to the NMRSARL within this time period, the transmission of novel PVL-positive and PVL-negative ST5-MRSA lineages in Irish hospitals was investigated. Forty-seven MRSA isolates recovered from nine separate Irish hospitals between 2013 and 2020 underwent WGS analysis (Table 4.2). This included the 14 previously described PVL-positive t002/ ST5-MRSA-IVc outbreak-associated isolates from H3 and 26 PVL-negative t311/ST5-MRSA-V isolates from five separate Irish hospitals including H3. Seven other isolates including a PVL-positive t311/ST6045-MRSA-V and six PVL-negative t002/ST5-MRSA-IVc/IVg isolates recovered during this period (2013–2019) were also included for comparative purposes. Isolate ST6045 was included due to the allelic profile similarities between ST5 (1–4–1–4–12–1–10) and isolate ST6045 (1–4–359–4–12–1–10). The 14 PVL-positive t002/ST5-MRSA-IVc isolates were recovered between 2018–2020 from patients in the maternity ward (*N*=9), the SCBU (*N*=3), the outpatient department (*N*=1) and the paediatric emergency department (*N*=1) of H3. Where metadata was available (23/26; 88.5%), the PVL-negative t311/ ST5-MRSA-V isolates were recovered between 2015–2019 from patients in the general wards (*N*=19), the SCBU (*N*=2), emergency department (*N*=1) and the high dependency unit (*N*=1) of H1, H3, H6, H13 and H14. The comparator isolates were recovered from the H3 maternity unit (*N*=1), the gynaecology ward (*N*=1) and general wards (*N*=5) of H4, H9, H13 and H21.

All 14 PVL-positive t002/ST5-MRSA-IVc isolates exhibited phenotypic resistance to ampicillin. The 26 PVL-negative ST5/t311-MRSA-V isolates exhibited resistance to a variety of antimicrobial agents including ampicillin (26/26; 100%), fusidic acid (26/26; 100%), ciprofloxacin (22/26; 84.6%), trimethoprim (22/26; 84.6%) and erythromycin (16/26; 61.5%) (Table 4.2). Resistance to tetracycline and rifampicin was also observed in a few isolates (4/26; 15.4%) (Table 4.2). Genotypic characterisation also confirmed that all the PVL-negative t311 isolates harboured the *fusC* gene which encodes resistance to fusidic acid. The PVL-negative t311/ST6045-MRSA-V comparator isolate exhibited a similar resistance profile to the t311/ST5-MRSA-V isolates including resistance to ampicillin, fusidic acid (including carriage of the *fusC* gene), erythromycin, ciprofloxacin

and trimethoprim. One PVL-negative comparator t002/ST5-MRSA-IVc isolate exhibited phenotypic resistance to ampicillin and ciprofloxacin while the remaining comparator t002/ST5-MRSA-IVc/IVg isolates exhibited resistance to ampicillin (5/5; 100%), erythromycin (1/5; 20%), ciprofloxacin (1/5; 20%) and chloramphenicol (1/5; 20%).

4.3.5 Phylogenetic analysis of Irish ST5-MRSA isolates

To investigate the genetic relatedness of these novel ST5-MRSA strains circulating in Irish hospitals during the same period, all 47 study isolates and comparators were subjected to WGS-based phylogenetic analyses. For this investigation, the pre-defined thresholds for inferring epidemiological relationship between *S. aureus* isolates were deemed appropriate (Schürch *et al.,* 2018); ≤15 cgSNPs or ≤24 cg/wgMLST allelic differences was considered as indicating close relatedness and indicative of recent transmission.

4.3.5.1 Core-genome SNP analysis

The cgSNP analysis based on 2,179 SNPs revealed distinct lineage-specific clades of ST5-MRSA. The majority of the H3 outbreak-associated PVL-positive t002/ST5-MRSA-IVc isolates (13/14; 92.9%) grouped into one clade, termed Clade I (Fig. 4.7) and exhibited a pairwise SNP-distance median of 4 (average: 4; range: 0–8) from one another. Despite the genotypic similarities between the PVL-negative t311/ST5-MRSA-V+*fus* study strains, the isolates grouped into two separate clades, termed Clade II and III (Fig. 4.7). Clade II isolates had a median of 3 (average: 3; range: 0–4) SNPs between one another and consisted of only isolates recovered from H6. The remaining PVL-negative t311/ST5-MRSA-V isolates (22/26; %) recovered from four separate hospitals grouped into Clade III alongside the PVL-negative t311/ST6045-MRSA-V comparator isolate (red asterisk in Fig. 4.7). Clade III isolates had a median of 87 (average: 75; range: 1–137) SNPs between one another and the ST6045 comparator exhibited between 62–107 cgSNPs to its neighbouring ST5 Clade III isolates. The remaining comparator isolates (6/7; 85.7%) formed an outgroup at the base of the tree (Fig. 4.7). Clade II differed from Clade III by a median of 476 (average: 477; range: 460–497) SNPs while Clade I differed from Clade II and III by a median of 586 (average: 585; range: 562–594) and 607 (average: 606; range: 572–629) SNPs, respectively. These findings indicate that numerous distinct ST5-MRSA lineages have been independently introduced into Ireland and are all disseminating widely within different Irish hospitals.

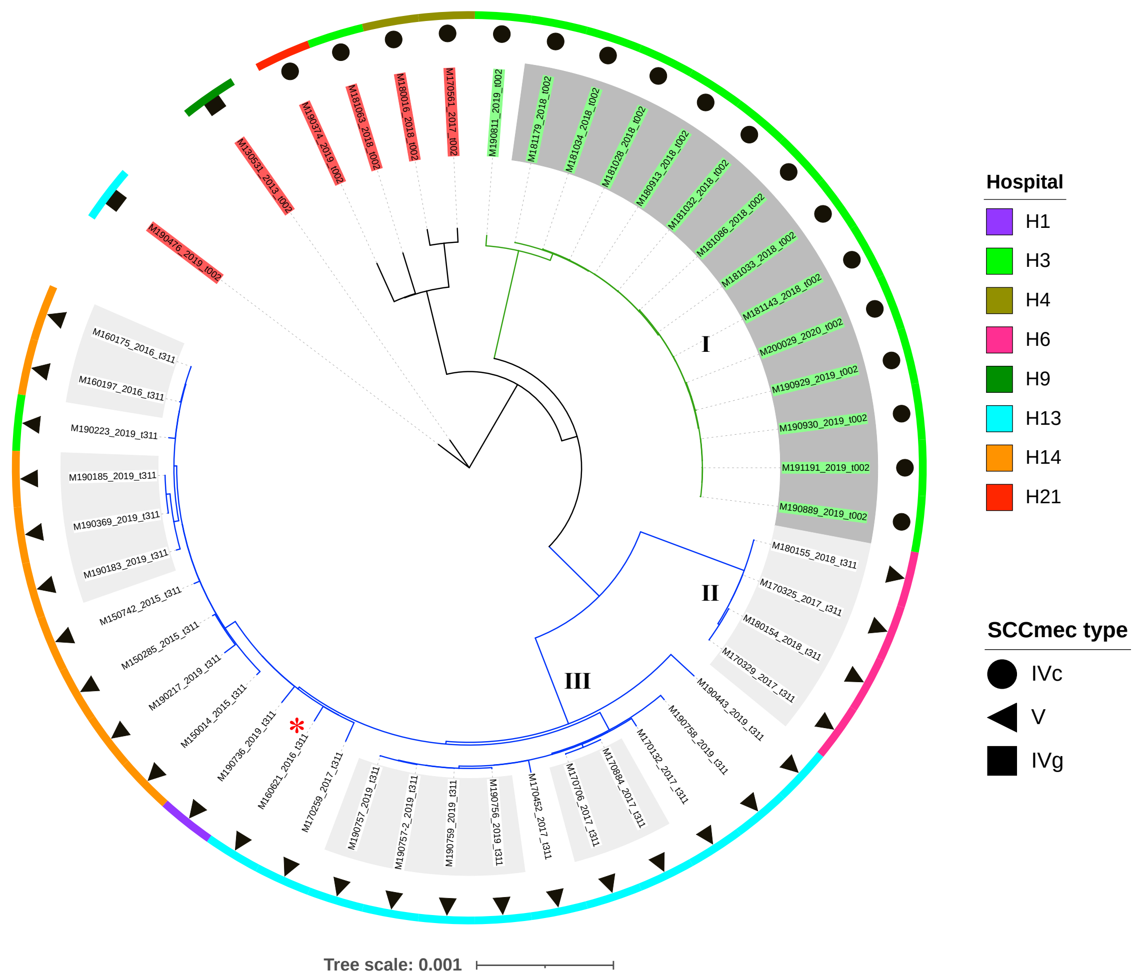

Figure 4.7. Maximum likelihood tree (MLT) based on phylogenetic analysis of 2,179 core-genome single nucleotide polymorphisms (cgSNPs) for 14 Panton-Valentine leukocidin (PVL)-positive t002/ST5-MRSA-IVc isolates, 26 PVL-negative t311/ST5-MRSA-V isolates and seven additional PVL-negative t002/ST5-MRSA-IVc/Ivg (*N*=6) and t311/ST6045-MRSA-V (*N*=1) comparator isolates. Isolates were recovered from eight separate hospitals across Ireland between 2003–2020. Separate node colours/shapes represent hospital and SCCmec types as indicated in the legend. Green branches represent the PVL-positive t002/ST5-MRSA-IVc isolates forming Clade I. Blue branches represent the PVL-negative t311/ST5-MRSA-V isolates forming Clades II and III. Labels for the comparator isolates which separate away from the main branch are highlighted in red. Isolate names, year of recovery and *spa* types are all indicated in the branch labelling. Clusters containing closely related isolates that differed by ≤15 cgSNPs are shaded in grey. The t311/ST6045-MRSA-V comparator which grouped among the t311/ST5-MRSA-V isolates is indicated by a red asterisk. The epidemiological and genotypic information for each isolate investigated is provided in Table 4.16. The

cgSNP-based MLT was constructed using IQ-TREE v2.2.0 (http://www.iqtree.org) and the phylogenetic tree was visualised and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

In general, the isolates grouped according to the hospital in which they were recovered, with numerous hospital-specific clusters of closely related isolates that differed by ≤15 cgSNPs observed on the MLT (shaded in grey in Fig. 4.7). Notably, a PVL-negative t311/ST5-MRSA-V isolate (M190223) recovered from H3 in 2019 grouped closely with two other PVL-negative t311/ST5-MRSA-V isolates (M160175 and M160197) recovered from H14 in 2016 exhibiting only 17–19 SNPs to these isolates. Despite the three-year gap in recovery date, the close phylogenetic relationship between these isolates is indicative of transmission of this particular strain between H3 and H14, possibly by frequent transfer of patients or HCWs between the two hospitals.

4.3.5.2 Core/whole-genome MLST analysis

The cgSNP analysis described above revealed numerous clusters (≤15 cgSNPs) of closely-related PVL-negative ST5-MRSA isolates which were all recovered from separate patients within these hospitals. This is indicative of further ST5-associated outbreak events in Irish hospitals within this time-period, separate to the previously described H3 PVL-positive t002/ST5-MRSA-IVc outbreak. These suspected outbreaks were further investigated using cg/wgMLST tools. The cgMLST-based and wgMLST-based MSTs constructed also revealed that the PVL-negative t311/ST5-MRSA-V isolates formed hospital-specific clusters (C1–C3) of closely related isolates (≤24 allelic differences) with no evidence of ST5-MRSA transmission between hospitals in most cases (Fig. 4.8a and 4.8b). PVL-negative t311/ST5-MRSA-V isolates (M150014, M150285, M150742, M160175, M160197, M190217, M190185, M190369 and M190183) from H14 clustered into C1, PVL-negative t311/ST5-MRSA-V isolates (M170452, M170884, M170132, M170706, M190756, M190757, M190757-2, M190758 and M190759) from H13 clustered into C2 while PVL-negative t311/ST5-MRSA-V isolates (M170325, M170329, M180154 and M180155) from H6 clustered into C3. The previously described PVL-positive t002/ST5-MRSA-IVc outbreak isolates (M180913, M181028, M181032, M181033, M181034, M181086, M181143, M181179, M190811, M190889, M190929, M190930, M191191 and M200029) clustered together into C4.

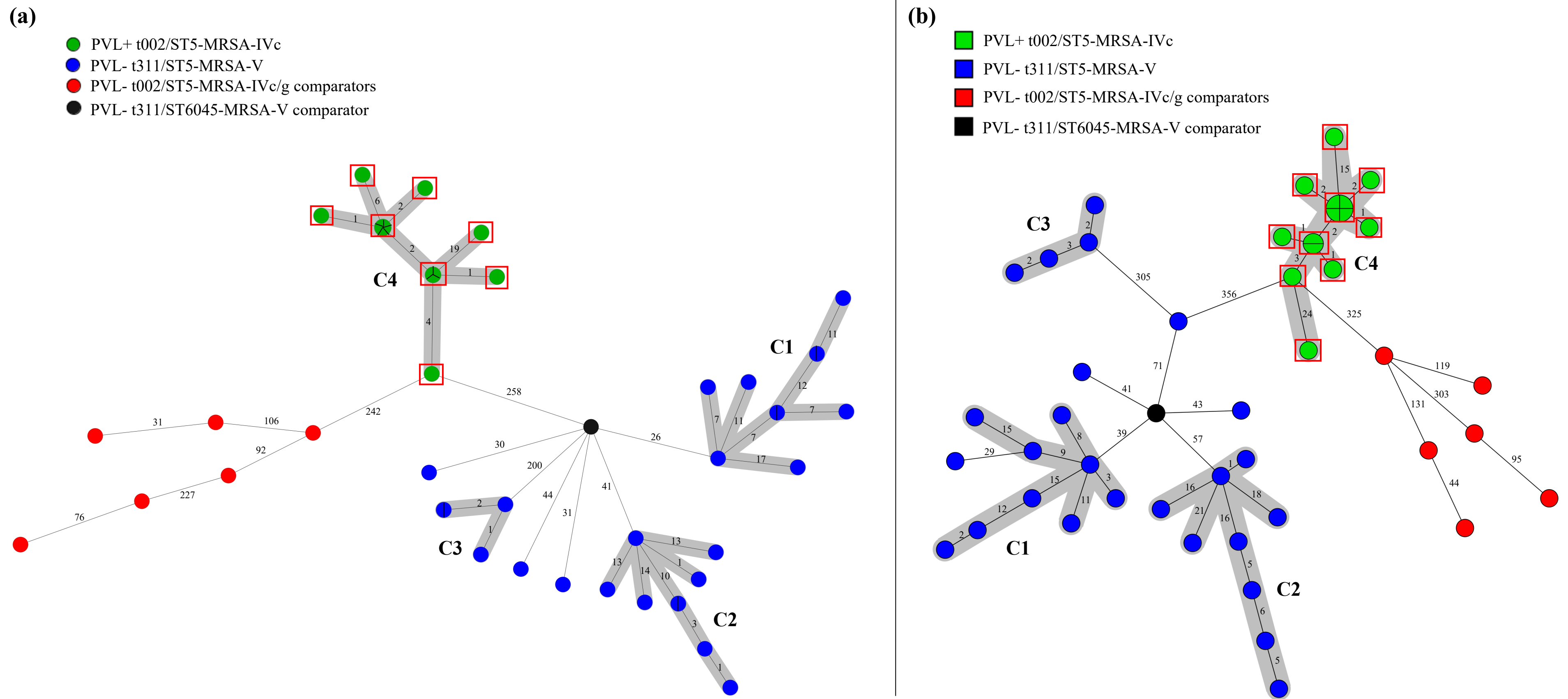

Figure 4.8. Minimum spanning trees (MSTs) based on (a) core-genome multi-locus sequencing typing (cgMLST) and (b) whole-genome multi-locus sequencing typing (wgMLST) analysis of 14 Panton-Valentine Leukocidin (PVL)-positive t002/ST5-MRSA-IVc and 26 PVL-negative t311/ST5-MRSA-V isolates and seven PVL-negative t311/t002 ST5/ST6045-MRSA-IVc/IVg/V comparator isolates recovered from Irish hospitals between 2013 and 2020. In each MST, MRSA isolates belonging to each group stated are indicated by separate colours. Closely related clusters

of isolates with ≤24 cg/wgMLST allelic differences are outlined with grey shadowing. Clusters of closely related isolates are indicated as C1–C4. The numbers on each branch indicate the numbers of cg/wgMLST allelic differences detected between neighbouring isolates. A red square is used to indicate a PVL-positive isolate. The epidemiological information for each isolate investigated is shown in Table 4.6.

Within both MSTs, the H3 t311/ST5-MRSA-V isolate (M190223) clustered into C1 with the H14 t311/ST5-MRSA-V isolates (Fig. 4.8a and 4.8b). Additionally, the t311/ST6045-MRSA-V comparator isolate (M160621) exhibited only 26 cgMLST (39 wgMLST) allelic differences to the closest neighbouring t311/ST5-MRSA-V isolate (M150285) within C1 and 41 cgMLST (57 wgMLST) allelic differences to the closest neighbouring t311/ST5-MRSA-V isolate (M170884) within C2. In contrast, the six remaining ST5-MRSA comparator isolates (M130531, M170561, M180016, M181063, M190374 and M190476) separated from the clusters and exhibited a minimum of 242 cgMLST (325 wgMLST) allelic differences to the closest neighbouring cluster (C4) (Fig. 4.8a and 4.8b).

Collectively, the findings of this SNP/MLST-based phylogenetic analysis indicates the occurrence of multiple outbreaks caused by distinct ST5 lineages within different Irish hospitals concurrently. The similarities in *spa* types and period of recovery between the investigated PVL-positive and PVL-negative t002/t311 ST5 isolates appears to be purely coincidental with no other links observed between the groups of isolates. The increase in the proportion of novel ST5-MRSA isolates being submitted to the NMRSARL in recent years is most likely due to the occurrence of several separate unrelated outbreaks within several hospitals.

4.4 Discussion

The increasing prevalence of PVL-positive MRSA isolates from non-BSIs and hospital outbreaks both in Ireland and internationally is concerning. A general population study of MRSA in Sri Lanka by McTavish *et al*., 2019, revealed a highly prevalent PVL-positive CC5-MRSA-IVc lineage in a Sri Lankan hospital and also identified it in the UK and Australia. Chapter 3 of this study also described similar isolates from 13 patients recovered during a protracted Irish maternity unit hospital outbreak between 2018–2020. Consequently, this study aimed to further characterise the international dispersal of this CA-MRSA clone based on its molecular epidemiology and phylogenetic diversity. To achieve this, a comparative genomic analysis of 214 Sri Lankan clone and 52 comparator isolates from Ireland and 14 other countries was performed, with isolate recovery spanning almost two decades.

The phylogenetic analysis revealed that the vast majority of Sri Lankan clone isolates group into a single primary clade and that the clone is relatively homogenous compared with other PVL-positive CA-MRSA clones that have diverged more significantly over time (Challagundla *et al.,* 2018). Greater diversity maybe revealed in future studies with more disparately recovered isolates. Additionally, the segregation of Sri Lankan clone isolates largely into country-specific RIGs probably reflects local transmission and clonal evolution. Some Danish isolates in country-specific RIGs also formed household-specific clusters (RIGs 4, 9–10, 13, 18–21, 24 and 29) (Table 4.6 and Fig. 4.5). In some cases, different members of the same household presented with either carriage or infection. Furthermore, two isolates recovered from separate patients within the same Danish hospital clustered together and were closely related (RIG–30) (Table 4.6 and Fig. 4.5). These findings highlight the significance of CA-MRSA transmission in both community and hospital settings. Only limited inter-country dissemination of closely related Sri Lankan clone isolates was detected (RIG-3, RIG-11 and RIG-14) (Table 4.6), although this possibly reflects the limited collection of isolates available for investigation.

The Sri Lankan clone isolates investigated were ST5, predominantly *spa* type t002 or closely related *spa* types and harboured a relatively small number of antimicrobial-resistance genes. DNA microarray and WGS data analyses revealed variable IEC gene

cluster (*sea*/*sak*/*scn*/*sep*/*chp*) and plasmid-encoded enterotoxin genes (*sed*/*sej*/*ser*) detection, while the majority of other molecular characteristics were highly conserved. Although the predominant IEC variant among the Sri Lankan clone isolates in this study was type G (91.6%), six other IEC types were detected including IEC-types D/E/F and three non-typeable variants. Additionally, while the *sed/sej/ser* enterotoxin genes were absent in a small number of isolates investigated (6.5%), the majority harboured these genes. Variation in IEC types and enterotoxin genes probably reflects loss/gain of β-haemolysin converting bacteriophages encoding IEC genes and *sed*/*sej*/*ser*-encoding plasmids (Varshney *et al.,* 2009; Xia and Wolz, 2014). The prevalence of the MDR PVL-negative European CC1-MRSA-IV clone in Ireland exemplifies the importance of mobile genetic elements in the successful dissemination of emerging MRSA clones (Challagundla *et al.,* 2018; Earls *et al.,* 2019; Lindsay *et al.,* 2012; Watkins *et al.,* 2012). Earls *et al*., 2021, recently described the emergence of a distinct variant of the European CC1-MRSA-IV harbouring conjugative plasmid-encoded *ileS2*/*mupA* and *qacA* genes which confer high-level mupirocin and chlorhexidine resistance. The study reported on the subsequent rapid expansion of this CC1-MRSA-IV clone across Europe in the late 1990s (Earls *et al.,* 2019; Earls *et al.,* 2021). The European CC1-MRSA-IV is now the predominant endemic CC1-MRSA clone in Ireland, and the variant has been associated with community transmissions and multi-hospital outbreaks (Earls *et al.,* 2021).

Periodic replacement of predominant MRSA clones in Irish hospitals is well-documented (Kinnevey *et al.,* 2014; Shore *et al.,* 2005), thus the recovery of the Sri Lankan clone in 10 Irish hospitals over a nine-year period (2013–2022) is concerning. Additionally, the absence of the *bbp/sdrE* calcium binding cell surface protein encoding gene within only the Irish maternity unit hospital outbreak-associated Sri Lankan clone isolates is interesting. This outbreak-specific variation very likely reflects local loss of the gene as other Irish isolates investigated harboured the gene. Previous reports have demonstrated the distinct roles played by each *sdr MSCRAMM (*microbial surface components recognizing adhesive matrix molecules) surface protein in *S. aureus* pathogenicity (Liu *et al.,* 2015; Sabat *et al.,* 2006). Other studies have also reported that expression of the *bbp* gene may correlate with genes for methicillin-resistance and PVL, suggesting that this variation in the Irish maternity unit outbreak isolates may be somewhat significant (Wiśniewska *et al.,* 2014). Further research is required to continue monitoring the local evolution of this clone as it becomes more prevalent.

Although this study has provided the most extensive insight to date into the population structure, international dissemination and diversity of this novel PVL-positive CA-MRSA CC5 clone, there were some limitations to the study in regard to the epidemiological and clinical data available. Limited Sri Lankan clone isolates/WGS datasets were recovered following comprehensive literature and WGS database searches making it difficult to assess its true prevalence. Additionally, the majority of Sri Lankan clone isolates available for investigation were recovered mainly from Europe and the Middle East, making it difficult to fully determine the global burden of this clone. Another possible limitation is the timespan in which isolates were recovered. The earliest Sri Lankan clone isolate available for this study was recovered in 2005, indicating that predecessor strains belonging to this lineage could potentially have been missed. Furthermore, historical and contemporary data on MSSA progenitor populations is limited in most MRSA lineages (Steinig *et al.,* 2021), including the Sri Lankan clone. Despite the limited epidemiological data available, the present study also found associations between some of the patient samples with countries in South-East Asia, specifically Sri Lanka, which further supports prior research by McTavish *et al*., 2019, linking the possible origins of this PVL-positive CC5-MRSA clone with this geographical region. Future investigations require a more comprehensive isolate collection with good quality metadata, including potential progenitor MSSAs from more numerous and disparate regions.

This study highlighted the wide-spread transmission of not only a novel PVL-positive ST5-MRSA lineage in Irish hospitals, but also PVL-negative ST5-MRSA. Phylogenetic analysis of the PVL-negative t311/ST5-MRSA-V+*fusC* isolates recovered from numerous separate Irish hospitals during the same period of time revealed the association of these isolates with transmission events and multi-hospital outbreak clusters. The circulation of PVL-positive and PVL-negative ST5-MRSA within hospital settings is now commonplace in Ireland, however the spread of ST5 CA-MRSA lineages harbouring the SCC*mec* V+*fusC* element is interesting as it is not well-described in the literature. The majority of ST5 lineages previously described in Ireland have primarily been associated with SCC*mec* IV/II elements, including HA ST5-MRSA-II (USA100) and ST5-MRSA-IV (USA800) (Broderick *et al.,* 2021; Monecke *et al.,* 2011). In the present study, a comparator strain identified as t311/ST6045-MRSA-V+*fusC* exhibited only 26 cgMLST (39 wgMLST) allelic differences to a neighbouring t311/ST5-MRSA-

V+*fusC* isolate. The cgSNP analysis also revealed that this strain grouped into a clade comprised of t311/ST5-MRSA-V+*fusC* isolates. This close phylogenetic relatedness observed between isolates assigned to the same *spa* type but separate MLST groups demonstrates the advantages of employing multiple typing tools in conjunction for surveillance and transmission studies.

In conclusion, international and local surveillance of emerging MRSA clones is important for monitoring transmission. The association of a novel PVL-positive clone with both community and hospital settings in 12 countries spanning 17 years reflects its emergence internationally. The circulation of novel PVL-positive and PVL-negative CC5-MRSA lineages in Irish hospitals also highlights the diversity in MRSA populations being introduced into Ireland, very likely through migration and international travel.

Chapter 5

A distinct Panton-Valentine leukocidin (PVL)-encoding bacteriophage remnant harboured by the CC5/ST5-MRSA-IVc Sri Lankan clone investigated by WGS

5.1 Introduction

Staphylococcus aureus is a highly clonal species as evidenced by its well-conserved core DNA region which makes up approximately 75% of its entire genome (Turner *et al.,* 2019). Much of its diversity is largely determined by MGEs in the accessory genome (Turner *et al.,* 2019). This primarily includes bacteriophages (also known as phages), plasmids, transposons and pathogenicity islands, which often encode virulence, toxin and antimicrobial resistance genes and are acquired through horizontal transfer (Malachowa and DeLeo, 2010). In general, bacteriophages are considered to be one of the most important MGEs carried by *S. aureus* and are closely linked with staphylococcal diversity and evolution (Malachowa and DeLeo, 2010; Xia and Wolz, 2014). These small intra-cellular bacterial viruses are commonly classified into two separate groups based on their life cycles (Howard-Varona *et al.,* 2017). Lytic (virulent) phages typically infect the bacterial host, replicate and release progeny particles through lysis of the host (Howard-Varona *et al.,* 2017). Lysogenic (temperate) phages on the other hand, integrate into the host bacterial genome as a prophage (Howard-Varona *et al.,* 2017). Integration of temperate phages into the chromosome of *S. aureus* typically occurs through site- and orientation-specific recombination between an attachment site sequence present in the bacterial chromosome (*attB*) and bacteriophage genome (*attP*) (Fig. 5.1) (Leinweber *et al.,* 2021). Different temperate phages integrate into different regions of the bacterial chromosome based on the location of the attachment site sequence (Leinweber *et al.,* 2021). The insertion of the linear phage genome into the bacterial chromosome is mediated by a phage-encoded integrase protein (Int), resulting in the duplication of the attachment site sequence at the ends of the integrated lysogenic phage genome (Fig. 5.1) (Leinweber *et al.,* 2021). Temperate phages within the chromosome of *S. aureus* mediates the acquisition of a range of virulence factors and immune evasion cluster genes (Rohmer and Wolz, 2021; Rotman *et al.,* 2020).

Most *S. aureus* strains harbour multiple temperate phages integrated into their genome at the same time (Ingmer *et al.,* 2019; Rohmer and Wolz, 2021). These commonly include the highly prevalent β-haemolysin-converting prophages, present in virtually all *S. aureus* strains recovered from humans (Wamel *et al.,* 2006). In contrast, virtually all animal strains of *S. aureus* lack β-haemolysin-converting prophages (Howden *et al.,* 2023). β-haemolysin-converting phages integrate into the bacterial chromosomally-encoded β-haemolysin gene, *hlb,* resulting in insertional inactivation of *hlb*. These

phages carry combinations of *S. aureus* Immune Evasion Cluster (IEC) genes (*sak*/*scn*/*chp*/*sea*) within the phage genome (Fig. 1.4) and promote immune evasion (Rohmer and Wolz, 2021; Wamel *et al.,* 2006). Such phages are known as converting phages as they mediate the gain and loss of several functions such as the loss of the ability to express β-haemolysin and gain of the ability to express proteins such as staphylokinase, enterotoxin A and other IEC proteins. Similarly, integration of prophages into lipase genes within *S. aureus* is also common, as seen with the ϕ11.1 prophage which lysogenises the chromosomal lipase gene, *geh* (Byang *et al.,* 2022). Lysogeny of ϕ11.1 into *geh* results in insertional inactivation of glycerol ester hydrolase production (Byang *et al.,* 2022; Xia and Wolz, 2014). This disruption through phage integration is often referred to as negative conversion (Xia and Wolz, 2014), and is thought to confer protection against lipid toxicity to the bacteria (Byang *et al.,* 2022).

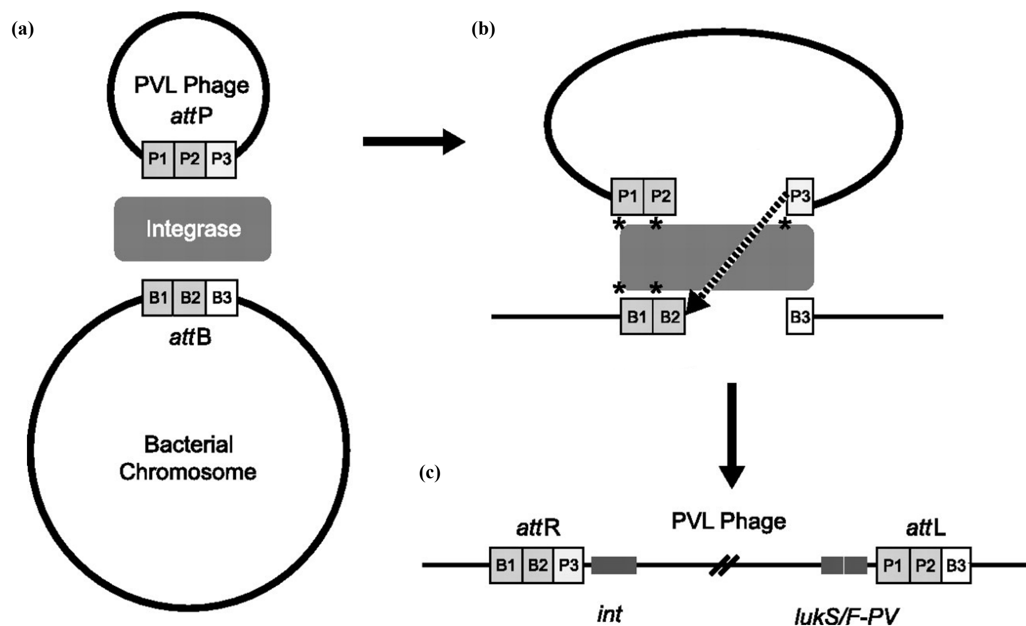

Figure 5.1. Integration of a PVL-encoding bacteriophage into the *S. aureus* chromosome. Insertion occurs via the *attP* and *attB* sites and forms two new attachment sites, *attR* and *attL*. P1/P2/P3; phage binding sites, B1/B2/B3; chromosome binding sites. Adapted from Boakes *et al.,* 2011.

The genes which encode for the PVL cytotoxin (*lukF/S-PV*) are also typically located in the genomes of lysogenic prophages (Fig. 5.1) (Boakes *et al.,* 2011). To date, several distinct icosahedral-head or elongated-head PVL-encoding phages have been described in *S. aureus*, including φPVL, φ108PVL, φSLT, φSa2USA, φSa2958 and φ5967PVL (Fig. 5.2) (Boakes *et al.,* 2011; Prabhakara *et al.,* 2013). The genomes of these phage are approximately 45 kb in size and are commonly organised into four main functional modules including lysogeny, DNA replication/transcription, head and tail packaging and lysis (Coombs *et al.,* 2020). The *lukF/S-PV* toxin genes are typically located adjacent the well-conserved lysis module, with most of the genomic diversity among different PVL-encoding phages occurring in the DNA processing and morphogenesis modules (Fig. 5.2) (Coombs *et al.,* 2020).

As a result of its epidemiological association with numerous prominent clones, including the widely disseminated CA-MRSA USA300 and Bengal Bay clones, PVL has been extensively investigated in recent years for its role in fitness and transmissibility (Balaji *et al.,* 2017; Boakes *et al.,* 2011). Several studies have characterised many different PVL-encoding phages, particularly the φSa2USA phage (Fig. 5.2d) (Boakes *et al.,* 2011; Coombs *et al.,* 2020; Prabhakara *et al.,* 2013). Initially described in the hypervirulent ST8-MRSA-IV USA300, φSa2USA is now one of the most dominant PVL-encoding phage types associated with MRSA worldwide (Boakes *et al.,* 2011; Sanchini *et al.,* 2014). It is suggested that USA300 most likely acquired the φSa2USA phage from its MSSA ancestor (Coombs *et al.,* 2020). The dominant Western Australian ST93-MRSA-IV CA-MRSA clone (Queensland clone) is also known to have acquired φSa2USA/φSa2wa-st93 from an ancestral MSSA clone (Coombs *et al.,* 2020; Coombs *et al.,* 2012). Similarly, the well-dispersed ST1-MRSA-IV CA-MRSA USA400 clone also harbours a PVL phage (φSa2MW) with close structural similarities to the USA300 associated φSa2USA (Zhang *et al.,* 2011). These findings indicate the occurrence of horizontal transfer of PVL phages between *S. aureus* strains (Coombs *et al.,* 2020). By contrast, genomic analysis of the Bengal Bay clone revealed a novel distinct PVL-encoding phage (Monecke *et al.,* 2013a; Prabhakara *et al.,* 2013). This ST772-MRSA-V CA-MRSA clone harbours the ΦIND772PVL phage, which contains parts of a *hlb*-converting phage translocated into its structural module, particularly the staphylococcal enterotoxin (*sea*) gene (Fig. 5.3) (Prabhakara *et al.,* 2013). Co-carriage of *pvl* and *sea* on the same prophage has not been reported in other *S. aureus* lineages or within any other

sequenced PVL-encoding phage (Balaji *et al.,* 2017). This highlights possible lineage-specific evolution of PVL phage within *S. aureus* clones.

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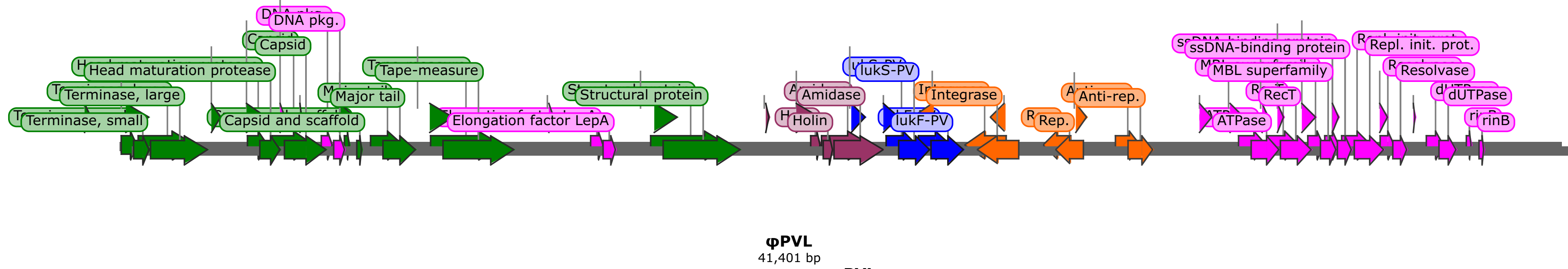
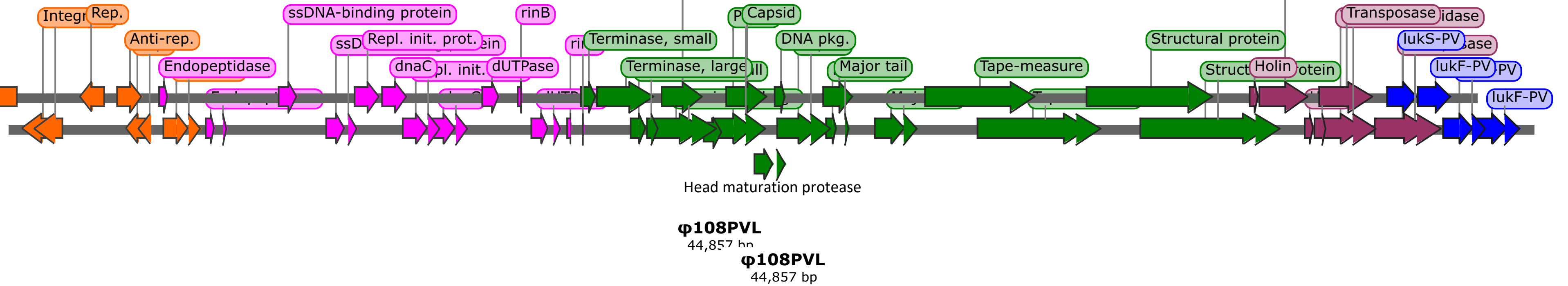
(a)



(b)



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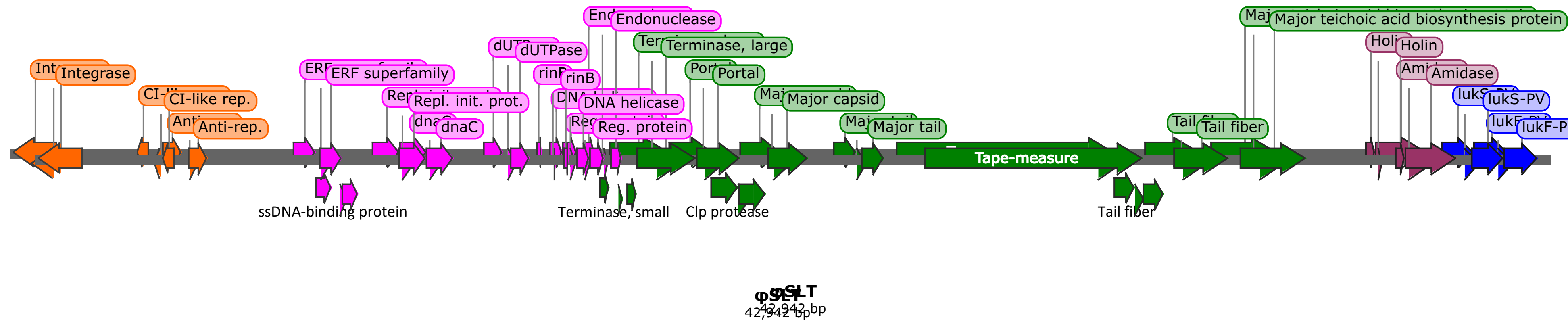
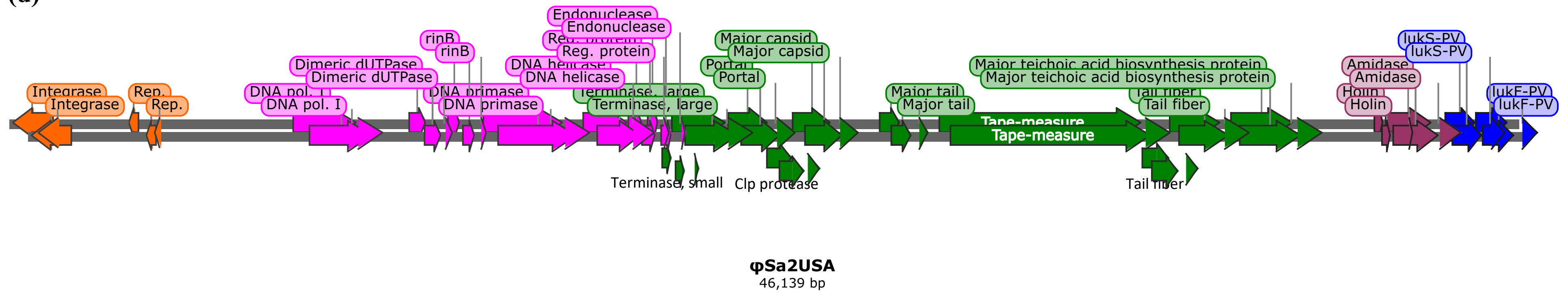
(c)



(d)



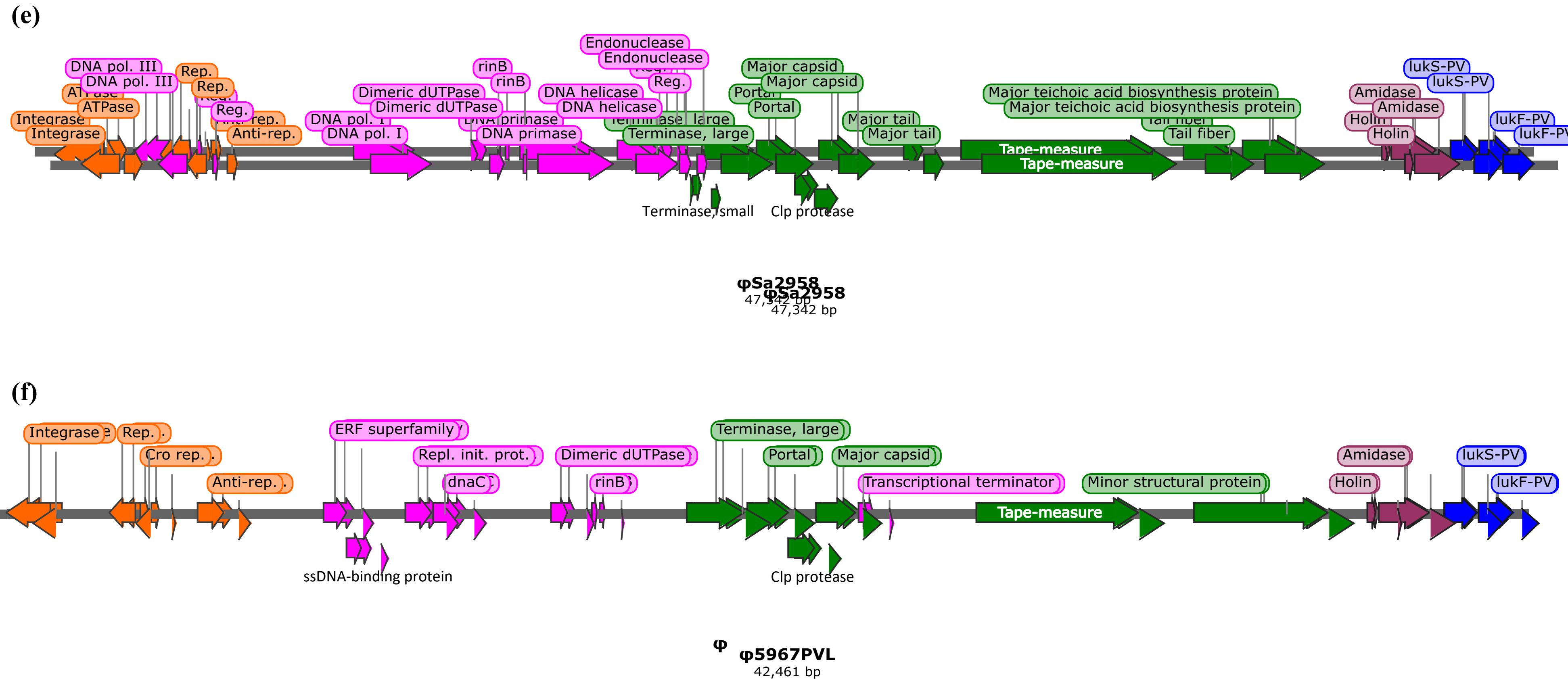


Figure 5.2. The genome map of representative *S. aureus* Panton-Valentine leukocidin (PVL)-encoding bacteriophages. Structural comparison of six distinct PVL phages; (a) φPVL, (b) φ108PVL, (c) φSLT, (d) φSa2USA, (e) φSa2958 and (f) φ5967PVL illustrated based on assembled genomes deposited in the GenBank database under accession Nos. AB009866.2, AB243556.1, AB045978.2, CP012120.2, AP009363.1 and AP011955.1, respectively. The typical components of the bacteriophage genome are highlighted by colour; lysogeny module (orange), DNA replication and

transcription genes (pink), head and tail packaging genes (green), lysis module (dark purple) and the *lukS/F*-PV region (blue). The assembled genomes were annotated using RAST v2.0 (https://rast.nmpdr.org) and visualised using SnapGene v6.0.6 (https://www.snapgene.com).

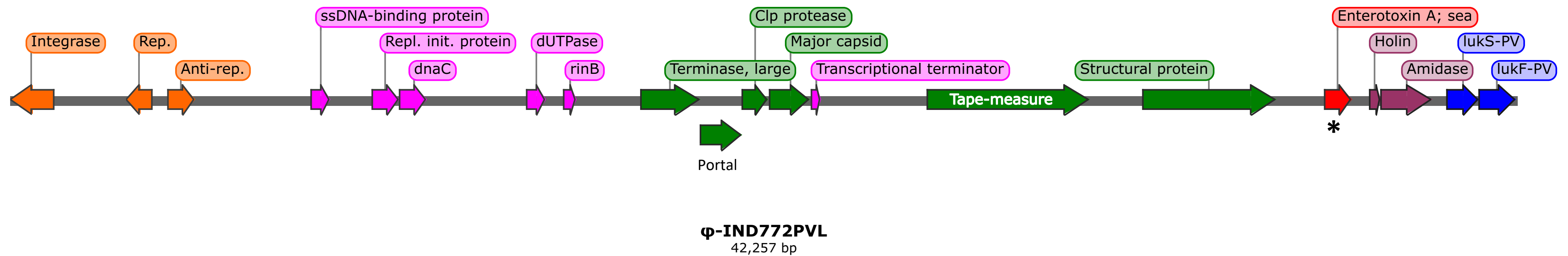


Figure 5.3. The genome map and structural organisation of a typical ΦIND772PVL bacteriophage harboured by the PVL-positive ST772-MRSA-V Bengal Bay clone. The typical components of the bacteriophage genome are highlighted by colour; lysogeny module (orange), DNA replication and transcription genes (pink), head and tail packaging genes (green), lysis module (dark purple) and the *lukS/F*-PV region (blue). The staphylococcal enterotoxin A (*sea*) gene typically encoded by β-haemolysin-converting prophages is highlighted in red and an asterisk indicates its novel location on the ΦIND772PVL phage. The publicly available reference genome of ΦIND772PVL (accession number CP010526.1) was annotated using RAST v2.0 (https://rast.nmpdr.org) and visualised using SnapGene v6.0.6 (https://www.snapgene.com).

This part of the present study sought to investigate the PVL phage type lysogenised into the genome of the PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone. Chapter 4 of this study provided the most comprehensive analysis of this emerging clone to date and identified Sri Lankan isolates within twelve separate countries over a 17-year period (2005–2022). To further characterise the Sri Lankan clone and identify its key molecular markers, a comparative analysis of the PVL gene sequences, the chromosome/phage integration sites and structural organisation of the PVL phage harboured by this clone was undertaken.

5.2 Materials and Methods

5.2.1 Isolate collection

A total of 285 samples including 128 clinical isolates and 157 WGS sequence datasets from 16 different countries recovered between 2003 and 2022 were investigated in this study. The collection comprised of PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates recovered from 13 countries between 2005–2022, PVL-positive and PVL-negative comparator CC5/ST5-MRSA–I/II/IVa/IVc/IVg/V isolates recovered between 2003–2021 and PVL-positive comparator CC5/ST5-MSSA isolates recovered between 2009–2019. Isolates were stored as described in Chapter 2, Section 2.2.1. A breakdown of the isolate collection is provided below.

5.2.1.1 Sri Lankan clone isolates

The 214 PVL-positive ST5-MRSA-IVc isolates recovered between 2005–2022 and defined as 'Sri Lankan clone' isolates in Chapter 4 were further investigated in this study (Table 4.1). This included isolates from Australia (*N*=1), Czech Republic (*N*=6), Denmark (*N*=66), Germany (*N*=20), Ireland (*N*=30), Kuwait (*N*=1), Norway (*N*=24), Saudi Arabia (*N*=4), Sri-Lanka (*N*=33), Sweden (*N*=2), United Arab Emirates (*N*=15) and United Kingdom (*N*=12).

Ten additional PVL-positive ST5-MRSA-IVc genomes identified by *in silico* PCR analysis of assembled *S. aureus* isolate genomes in the pubMLST database (see Section 5.2.5.2) were also included in the study (Table 5.1). The assembled genomes included one sample recovered from Luxembourg in 2009 and nine other samples with unknown country/year of recovery.

5.2.1.2 Comparator isolates

The 52 comparator PVL-positive (*N*=29) and PVL-negative (*N*=23) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V isolates recovered between 2003–2022, which were included in the study of the Sri Lankan clone in Chapter 4, were further investigated here (Table 4.1). These included isolates from Algeria (*N*=1), Czech Republic (*N*=2), Denmark (*N*=10), Germany (*N*=2), Ireland (*N*=17), Kuwait (*N*=1), Norway (*N*=12), Saudi Arabia (*N*=1), Senegal (*N*=2), Slovakia (*N*=1) and United Arab Emirates (*N*=3).

Nine additional PVL-positive ST5-MRSA-IVa (*N*=7) and ST5-MSSA (*N*=2) comparators submitted to the NMRSARL in 2019 (*N*=1) or identified by *in silico* PCR analysis of assembled *S. aureus* isolate genomes in the pubMLST database (*N*=8) (see Section 5.2.5.2) were also included in the study (Table 5.1). The assembled pubMLST genomes were samples with unknown country/year of recovery.

Table 5.1. Additional PVL-positive CC5-MRSA/MSSA isolates identified by *in-silico* PCR analysis

pubMLST ID[a]	Sample accession	Year	Country
PVL-positive ST5-MRSA-IVc			
9736	ERR211934	Unknown	Unknown
9743	ERR211966	Unknown	Unknown
12049	ERR212816	Unknown	Unknown
10203	ERR212871	Unknown	Unknown
25420	ERR540754	Unknown	Unknown
26854	ERR540938	Unknown	Unknown
28139	ERR541062	Unknown	Unknown
28178	ERR541068	Unknown	Unknown
30101	ERR714842	Unknown	Unknown
20953	SRR917592	2009	Luxembourg
PVL-positive ST5-MRSA-IVa			
9688	ERR204190	Unknown	Unknown
10741	ERR212783	Unknown	Unknown
9832	ERR212986	Unknown	Unknown
24398	ERR527305	Unknown	Unknown
30114	ERR702114	Unknown	Unknown
27603	ERR715326	Unknown	Unknown
25947	ERR737419	Unknown	Unknown
PVL-positive ST5-MSSA			
14797	ERR109505	Unknown	Unknown

[a] Assembled *S. aureus* isolate genomes in the pubMLST database harbouring the 9.6 kb PVL-encoding phage remnant

Abbreviations: PVL; Panton-Valentine leukocidin, SCC*mec*; staphylococcal cassette chromosome *mec*, MSSA; methicillin susceptible *Staphylococcus aureus*.

5.2.2 NMRSARL molecular characterisation

All Irish isolates included in the present study underwent species identification and phenotypic antimicrobial susceptibility testing at the NMRSARL as described in Chapter 2, Section 2.3. The NMRSARL also performed *spa* typing on these isolates as described in Chapter 2, Section 2.3.3.

5.2.3 Genomic DNA isolation

All clinical isolates underwent genomic DNA extraction as described in Chapter 2, Section 2.4.1.

5.2.4 Whole-genome sequencing

5.2.4.1 Second-generation Illumina short-read sequencing

All clinical isolates underwent short-read WGS as described in Chapter 2, Section 2.5.1.

5.2.4.2 Third-generation ONT MinION long-read sequencing

Where clinical samples were available, thirty-four clinical isolates representative of the different countries under investigation underwent long-read WGS as described in Chapter 4, Section 4.2.4.2.

5.2.4.3 Hybrid assembly

Isolates selected for hybrid assembly underwent genome scaffolding using paired-end short-read MiSeq sequences (Illumina) and long-read MinION sequences (ONT) as described in Chapter 4, Section 4.2.4.3.

5.2.5 Bioinformatic analyses

All study isolates underwent *de novo* assembly, *spa* type confirmation, SCC*mec* subtyping, MLST, *in silico* antimicrobial resistance profiling and virulence gene identification as described in Chapter 2, Section 2.6.

5.2.5.1 Bionumerics-based genome analysis tools

The search function within BioNumerics v8.0 (Applied Maths) was used to analyse and annotate the short-read assembled genomes of the study isolates. The BioNumerics v8.0 (Applied Maths) chromosome comparison tool was used for side-by-side *in silico* comparison of the genomes of different isolates.

5.2.5.2 In-silico PCR analysis and pubMLST search

The Sri Lankan clone isolates under investigation in this study harboured a PVL-encoding bacteriophage with a distinct genetic organisation (see Section 5.3.1). To identify additional strains carrying lysogenized PVL phages with a similar genetic organisation, all available 29,504 *S. aureus* assembled genomes in the pubMLST database (https://pubmlst.org) were screened using the pubMLST BLAST function (accessed 2nd Nov. 2022). An *in-silico* PCR primer design tool incorporated within SnapGene v6.0.6 (https://www.snapgene.com) was used to construct forward and reverse primers specific to the Sri Lankan clone PVL phage for the BLAST search (Table 5.2). The primers used targeted an 856 bp amplicon of all assembled genomes harbouring similar PVL-encoding bacteriophage as the Sri Lankan clone isolates. A 75 bp sequence "ΔPhage-seq probe" (Table 5.2) that spanned the chromosomal/5'-phage junction was used as a probe against the assembled genomes to confirm *in silico* PCR results.

5.2.5.3 Maximum likelihood analysis

A maximum likelihood phylogenetic analysis based on core-genome SNPs was performed for all 285 study isolates (224 Sri Lankan clone isolates and 61 comparators) as described in Chapter 4, Section 4.2.5.2.

Table 5.2. Primers and phage sequence search probe used for *in-silico* PCR analysis

Primer[a]	**Primer sequence**[c]
CC5Chromosome-F	5'-ATTCGATTGCACGTTCTG-3'
CC5Phage-R	5'-ACTTAACAGACGAGTTATTGCAC-3'
Probe[b]	**Probe sequence**
ΔPhage-seq probe	GAGTTGTTTAAAGGTATAAAATCTTTTATAGAAAAAGT GTATGATAGTATAAATAAAAAAGCTAAGCCTTGTATG

[a] Primers designed using SnapGene v6.0.6 (https://www.snapgene.com).

[b] 75 bp probe to confirm *in silico* PCR results.

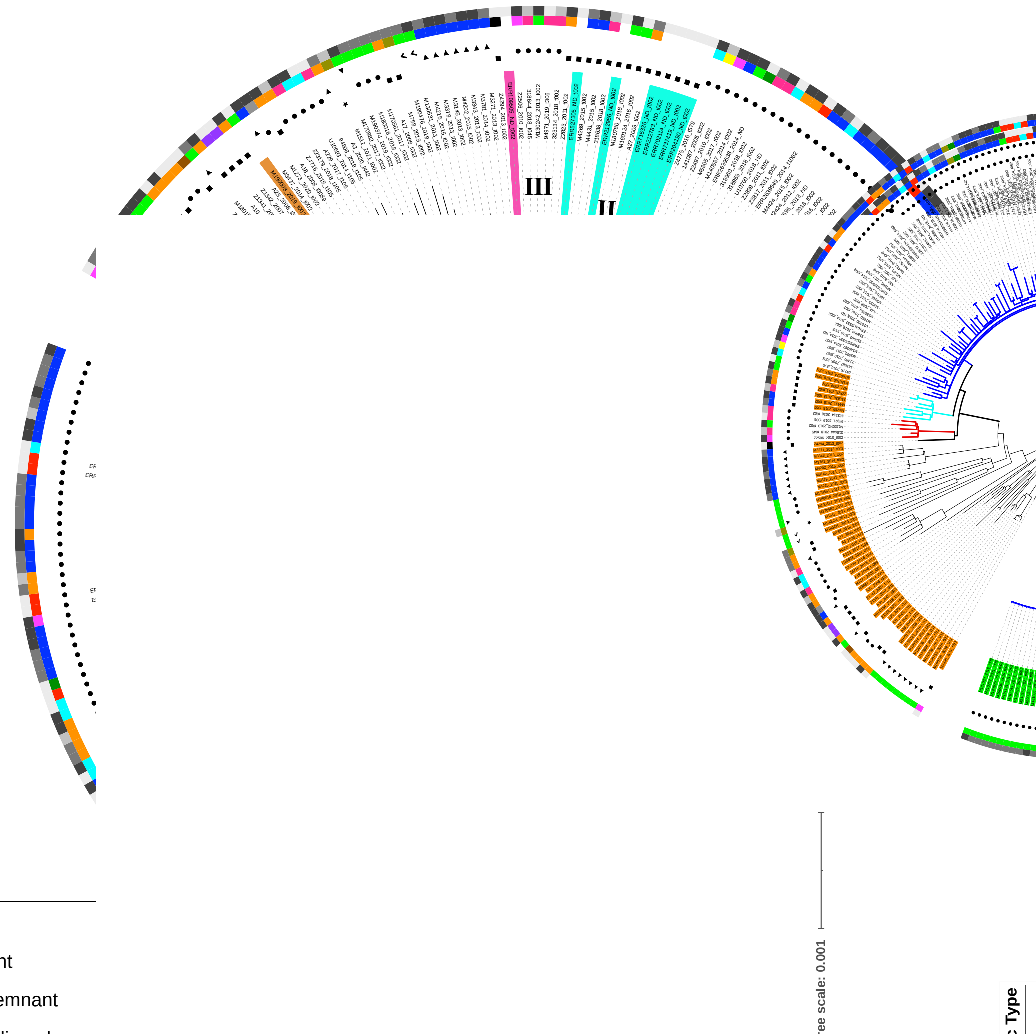
5.3 Results

5.3.1 Characterisation of the Sri Lankan clone PVL-encoding bacteriophage

To characterise the PVL-encoding bacteriophage associated with the Sri Lankan clone, the short-read assembled genomes of the 214 PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates recovered from twelve different countries between 2005 and 2022 were examined for *pvl*-associated bacteriophage DNA (Table 4.1). In addition, the short-read assembled genomes of the 29 PVL-positive comparator CC5/ST5-MRSA-IVa/IVc/V isolates recovered from eight different countries between 2003–2019 were also analysed for comparative purposes (Table 4.1). The genome analysis revealed that the Sri Lankan clone isolates (*N*=214) under investigation harboured the *lukF/S*-PV genes, the phage lysis genes encoding amidase and holin and partial remnants of the phage structural genes encoding the tail fiber and major teichoic acid biosynthesis protein. The genes associated with lysogeny, DNA replication/transcriptional regulation and packaging/structure, typically present in the lysogenised bacteriophage genome (Coombs *et al.,* 2020) were not detected. In addition, the seven PVL-positive comparator isolates which grouped into Clade II in the cgSNP analysis performed in Chapter 4, Section 4.3.2.1 also harboured a similar incomplete PVL-associated bacteriophage (Fig. 5.4). The PVL-positive comparator isolate Z4294 neighbouring Sri Lankan clone Clade III isolates in the cgSNP MLT also harboured an incomplete phage (Fig. 5.4). By contrast, the remaining PVL-positive comparators (*N*=21) which formed outgroup isolates in the cgSNP MLT all harboured complete PVL-encoding bacteriophages (Fig. 5.4).

To further investigate the unique genetic organisation of the PVL-encoding bacteriophage harboured by the Sri Lankan clone isolates, twenty-six representative Sri Lankan clone isolates (24 MLT Clade I and two MLT Clade III isolates; Chapter 4, Section 4.3.2.1) available for long- and short-read sequencing underwent hybrid-assembly (Fig. 5.4). Additionally, eight comparator isolates also underwent hybrid-assembly (five outgroup and three MLT Clade II comparators; Chapter 4, Section 4.3.2.1) (Fig. 5.4). The 26 hybrid-assembled Sri Lankan clone isolates were recovered between 2005 and 2021 from Czech Republic (*N*=1), Denmark (*N*=6), Germany (*N*=2), Ireland (*N*=8), Kuwait (*N*=1), Norway (*N*=3), Saudi Arabia (*N*=2), Sweden (*N*=1) and the UAE (*N*=2). The eight hybrid-assembled comparator isolates were recovered between 2003

and 2019 from Algeria (N=1), Germany (N=1), Ireland (N=1), Norway (N=3), and the UAE (N=2).



M181028_2018_t002
M181034_2018_t002
M181032_2018_t002
M180913_2018_t002
M181179_2018_t002
M181086_2018_t002
M200029_2020_t002
M210263_2021_t002
M190889_2019_t002
M181143_2018_t002
M191191_2019_t002
M190930_2019_t002
M190929_2019_t002
M210262_2021_t002
M181033_2018_t002
Additional isolates
Tree scale: 0.001
Country of Recovery
Algeria
Czech Republic
Denmark
Germany
SCCmec Type
I
II
IVa
IVc
IVg
V

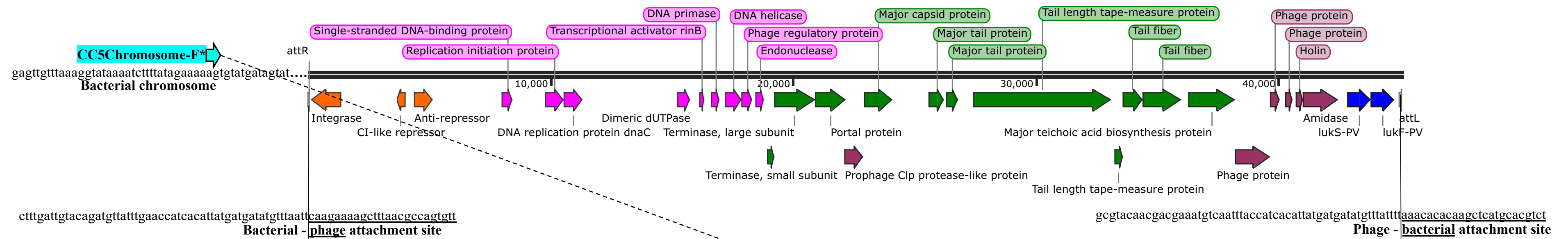
Figure 5.4. Maximum likelihood tree (MLT) based on phylogenetic analysis of 12,911 single nucleotide polymorphisms (SNPs) for 224 PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates (includes the 10 additional CC5/ST5-MRSA-IVc pubMLST isolates) and 61 PVL-positive (*N*=38) and PVL-negative (*N*=23) CC5/ST5-MSSA/MRSA-I/II/IVa/IVc/IVg/V comparator isolates (includes the 8 additional PVL-positive CC5/ST5-MRSA-IVa [*N*=7] and CC5/ST5-MSSA [*N*=1] pubMLST isolates and a PVL-positive CC5/ST5-MSSA comparator from the Irish National MRSA reference laboratory [*N*=1]). A total of 285 study isolates (Sri Lankan clone and comparator isolates) were recovered from 16 different countries between 2003–2022. Sri Lankan clone isolates were recovered from 13 countries across Europe, Asia, Australia and the Middle East between 2005–2022. Separate node colours/shapes represent country of recovery, sample types and SCC*mec* types as indicated in the legend. The blue branches represent PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates (*N*=219/224) forming Clade I. The green branches represent PVL-positive CC5/ST5-MRSA-IVa comparator isolates (*N*=14/61) forming Clade II. The red branches represent the remaining PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates (*N*=5/224) forming Clade III. The thick black branches represent a PVL-positive CC5/ST5-MSSA comparator isolate (pubMLST database ID 14797, Accession No. ERR109505) and a PVL-positive CC5/ST5-MRSA-IVa comparator isolate (*N*=2) branching out next to Clade III. The thin black branches represent the comparator outgroup isolates (*N*=45) which separate away from Clades I–III. Isolate names, year of recovery and *spa* types are all indicated in the branch labelling. All available 29,504 *Staphylococcus aureus* assembled genomes (accessed 16th Nov. 2022) in the pubMLST database (https://pubmlst.org) were screened using *in silico* PCR and isolates (*N*=18) carrying the PVL-phage remnant recovered from the database are highlighted in pink (PVL-positive CC5/ST5-MSSA; *N*=1), turquoise (PVL-positive CC5/ST5-MRSA-IVa; *N*=7) and blue (PVL-positive CC5/ST5-MRSA-IVc; *N*=10) within the MLT. A PVL-positive Irish MSSA isolate (M190008 highlighted in orange) harbouring an intact PVL-encoding phage was also included for comparative purposes. The limited available metadata on the 18 pubMLST isolates are provided in Table 5.1. The available genotypic information for the original 266 study isolates and all 19 additional isolates is provided in Tables 4.1 and 5.3, respectively. The cgSNP-based MLT was constructed

using IQ-TREE v2.2.0 (http://www.iqtree.org) and the phylogenetic tree was visualised and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

Closer examination using these hybrid-assembled genomes confirmed that all 26 Sri Lankan clone isolates lacked an intact lysogenized PVL-encoding phage (Fig. 5.5). However, the isolates harboured a chromosomal remnant encoding the *lukF/S-PV* genes as well as remnants of the phage structural and lysis genes (Fig. 5.5). In each case, the phage remnant was 9,616 bp, with an intact downstream attachment site (*attL*), but no upstream attachment site (*attR*). An identical phage remnant was observed in the three Clade II comparator isolates and the single PVL-positive ST5-MRSA-IVa comparator isolate (Z4294) next to Clade III (Fig. 5.4). The four remaining outgroup comparator isolates all harboured a complete bacteriophage genome of 44,823 bp which shared 99.99% sequence homology with the well-characterized PVL-encoding phage phiSa2wa (accession no. ON989481.1) (Fig. 5.5) (Coombs *et al.,* 2020).

A comparative sequence analysis of the 9,616 bp remnant harboured by the Sri Lankan clone isolates against a 44,823 bp reference phiSa2wa phage genome revealed that the phage remnant exhibited 100% sequence homology with the 3′ junction of phage phiSa2wa. Additionally, analysis of the chromosomal sequences upstream of the phage remnant revealed the absence of 614 bp of bacterial chromosomal sequence. This 614 bp sequence is typically present adjacent to the *attR* attachment site. This finding suggests that possible imprecise excision of the PVL phage also resulted in the deletion of a small fragment of the bacterial chromosome (Fig. 5.6). Furthermore, all isolates investigated by hybrid-assembly which harboured the PVL-phage remnant showed the presence of an identical chromosomal sequence adjacent to the remnant (Fig. 5.5). This suggests that the PVL-encoding phage remnant is a stable component securely integrated into the chromosome of the Sri Lankan clone. This stable integration of PVL could potentially be contributing to the widespread dissemination of the clone, as evidenced in this study where Sri Lankan clone isolates were recovered from twelve countries across Europe, Asia and the Middle East.

(a)



(b)

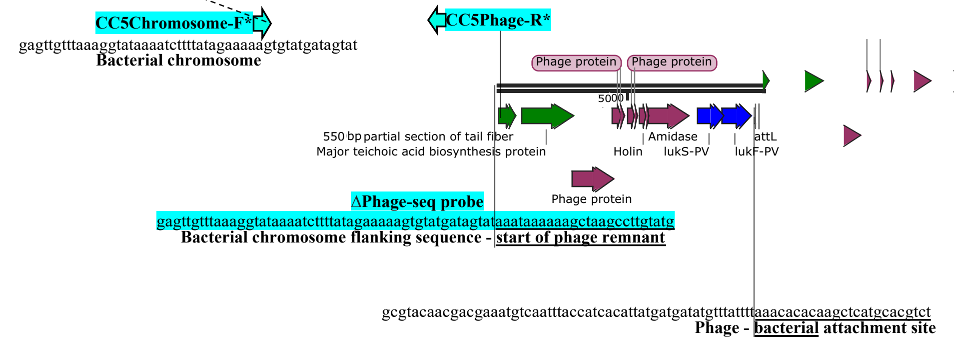


Figure 5.5. Comparison of the structural organisation of the Panton-Valentine leukocidin (PVL) *lukF/S*-PV genes and associated phage DNA sequences within the chromosome of a PVL-positive CC5/ST5-MRSA-IVc comparator isolate harbouring an intact lysogenised PVL-encoding bacteriophage genome, and a PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolate carrying a PVL-encoding phage remnant. The organisation of phage DNA regions was determined by hybrid assembly. (a) Comparator isolate 323179 (United Arab Emirates, 2018) harbouring a fully intact lysogenised PVL-encoding bacteriophage genome (~45,000 bp) with 99.99% sequence homology to the PVL-encoding phage phiSa2wa (accession no. ON989481.1). (b) Sri Lankan clone isolate M181179 (Ireland, 2018) harbouring a 9,616 bp PVL-encoding phage remnant

with 100% sequence homology to the 3' end junction of the phiSa2wa phage. The typical components of the bacteriophage genome are highlighted by colour; lysogeny module (orange), DNA replication and transcription genes (pink), head and tail packaging genes (green), lysis module (dark purple) and the *lukS/F*-PV region (blue). Attachment sites (*attR* and *attL*) are typically present at the proximal and distal junction ends of lysogenized prophage genomes integrated in the bacterial chromosome. The phage remnant probably arose from an imprecise excision event that resulted in loss of most of the phage genome and a 614 bp chromosomal DNA sequence upstream and including the *attR* site. In isolate M181179, the annotated bacterial chromosome flanking sequence and phage remnant' sequence are indicated. All available 29,504 *S. aureus* assembled genomes in the pubMLST database (https://pubmlst.org) (accessed 2nd Nov. 2022) were screened by *in silico* PCR using primers CC5Chromosome-F* (5'-ATTCGATTGCACGTTCTG-3') and CC5Phage-R* (5'-ACTTAACAGACGAGTTATTGCAC-3') indicated in (b) which yield an 856 bp amplimer with assembled genomes harbouring the 9.6 kb phage remnant. A 75 bp sequence "ΔPhage-seq probe" (highlighted in light blue in (b)) that spanned the chromosomal/5'-phage junction was used as an *in silico* probe against the assembled genomes to confirm *in silico* PCR results. The hybrid assembled genomes were annotated using RAST v2.0 (https://rast.nmpdr.org) and visualised using SnapGene v6.0.6 (https://www.snapgene.com).

>614 bp bacterial chromosomal sequence deletion
agataatgaaattttagaaattttcaaaaatatagatcacgacttcagagatggagtatctgaagaaatgatgaaacatttgaaagtagtgaaacagaatatagagcga
ataaaaaatcaaaatgatatttatggtaggcaaattgcagaaattagaagtattatgaaacaacaagatgcaacaattttagatgggaatttccaaattaattgtagcgg
cgaaaatatggtacagggtctagttataccttctaattatttaggaagaaaaatgaaaatattaaaagaccatatcgatgatggtattaaaaaaatagcagactatgttca
aagtatatatgatgaatatgcatcgaaaattgttgatgtaataaaatatttgattaatacaattcccaaaatacgtaagaatttaagacatgcaattgaaatgttaaatgtaa
aaaagaaagaatttttgtccctgattcctaatgtaacttgtaattatattaaaactaaattagaagaattagataatactttaggcaaatgggagccatttcttaatgatttaa
aagcagtgtcaccaattttagataaccatttagatgatattgttaagaacatgaagc

Figure 5.6. The 614 bp bacterial chromosome sequence deleted in Sri Lankan clone isolates. Analysis of the chromosomal sequences upstream of the phage remnant revealed the absence of 614 bp of bacterial chromosome, typically present adjacent to the *attR* attachment site. Analysis performed using SnapGene v6.0.6 (https://www.snapgene.com).

5.3.2 pubMLST search for isolates harbouring the 9,616 bp Sri Lankan clone PVL-phage remnant

To identify previously assembled genomes in the pubMLST database carrying a similar PVL-phage remnant to Sri Lankan clone isolates and determine how widely distributed this remnant is, further *in silico* analysis was performed. A pubMLST BLAST search was carried out using study-specific primer sequences and a phage sequence search probe (Table 5.2). The ∆Phage-seq probe was designed to cover the upstream junction between the bacterial chromosome and the phage remnant. A positive match indicated a truncated phage and a negative or incomplete match indicated an intact phage. Of the 29,504 pubMLST *S. aureus* genomes screened; 18 genomes were positive matches for carriage of this 9,616 bp PVL-phage remnant. Further analysis through SCC*mec*Finder and Ridom Seqsphere+ template tools confirmed that ten of these were PVL-positive CC5/ST5-MRSA-IVc, seven were PVL-positive CC5/ST5-MRSA-IVa and the remaining isolate was a PVL-positive CC5/ST5-MSSA strain.

The ten PVL-positive ST5-MRSA-IVc isolates were assigned to *spa* type t002 (*N*=8), t1154 (*N*=1) or were untypeable (*N*=1) (Table 5.3). Two closely related *spa* types were identified among the comparator isolates; t002 (*N*=6) and t045 (*N*=1) (Table 5.3). The CC5-MSSA isolate was assigned to *spa* type t002 (Table 5.3). Epidemiological information was available for only one isolate; a t002/ST5-MRSA-IVc isolate recovered in Luxembourg in 2009 (Table 5.1).

5.3.2.1 Phylogenetic analysis of Sri Lankan clone and pubMLST isolates

Despite the limited metadata available regarding year/country of recovery for the pubMLST isolates (Table 5.1), a phylogenetic analysis was performed against the 266 MRSA isolates described in Chapter 4 of this study. This analysis included the 18 pubMLST isolates, 214 PVL-positive CC5/ST5-MRSA-IVc Sri Lankan clone isolates recovered between 2005–2022 from 12 countries, 29 PVL-positive and 23 PVL-negative ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates (Fig. 5.4). Additionally, a PVL-positive t002/ST5-MSSA isolate (M190008) submitted to the NMRSARL from an Irish hospital in 2019 harbouring an intact PVL-encoding phage was also included for comparative purposes.

Table 5.3. Antimicrobial resistance and virulence-associated gene profiles of additional PVL-positive CC5-MRSA/MSSA isolates identified by *in-silico* PCR analysis (*N*=18) and from NMRSARL (*N*=1)

[a]

Isolates(*N*)	**Year(s) of isolation**	***spa*-ST-SCC*mec*(*N*)**	**AR genes[a](*N*)**	**PVL (+/-)**	**IEC Type(*N*)**	**Enterotoxin genes(*N*)**
ST5-MRSA-IVc						
10	2009 [1] ND [9]	t002-ST5-IVc [8] t1154-ST5-IVc [1] ND-ST5-IVc [1]	*blaZ, fosB, lmrP, mprF, sdrM, erm*(C) [5] *blaZ, fosB, lmrP, mprF, sdrM* [2] *fosB, lmrP, mprF, sdrM* [1] *blaZ, fosB, lmrP, mprF, sdrM, erm*(C), *tet*(K) [1] *aphA3, cat, blaZ, fosB, lmrP, mprF, sdrM, erm*(C), *tet*(K) [1]	+ [10]	G	*sed/sej/ser* [9] None [1]
ST5-MRSA-IVa						
7	ND [7]	t002-ST5-IVa [6] t045-ST5-IVa [1]	*blaZ, fosB, lmrP, mprF, sdrM, erm*(C) [4] *blaZ, fosB, lmrP, mprF, sdrM* [3]	+ [7]	G [6] None[b] [1]	*sed/sej/ser* [7]
ST5-MSSA						
2	2019 [1] ND [1]	t002-ST5 [2]	*blaZ, fosB, lmrP, mprF, sdrM, erm*(C) [1] *blaZ, fosB, lmrP, mprF, sdrM* [1]	+ [2]	A [1] G [1]	*sed/sej/ser* [2]

Genotypic information was extracted from whole-genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) genotyping.

[b] This isolate harboured an intact *hlb* gene and therefore did not have an IEC type.

Abbreviations: ND, not determined – information not available and *spa* types not determined using *in-silico* techniques on the available genomic sequence data; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; PVL, Panton-Valentine leukocidin; +, positive; -, negative; IEC, immune evasion cluster.

The cgSNP MLT analysis based on 12,911 SNPs revealed that the ten PVL-positive CC5/ST5-MRSA-IVc pubMLST isolates grouped into Clade I with 209/214 of the ST5-MRSA-IVc Sri Lankan clone isolates (shaded in blue in Fig. 5.4). These isolates exhibited a pairwise SNP-distance median of 105 (average: 110; range: 0–287) from one another. The seven PVL-positive CC5/ST5-MRSA-IVa pubMLST isolates grouped with the seven ST5-MRSA-IVa comparator isolates in Clade II (shaded in turquoise in Fig. 5.4) and exhibited a median of 85 (average: 78; range: 3–120) from one another. The PVL-positive CC5/ST5-MSSA pubMLST isolate branched out next to the Clade III ST5-MRSA-IVc Sri Lankan clone isolates and also neighboured the PVL-positive ST5-MRSA-IVa comparator isolate (Z4294) next to Clade III (shaded in pink in Fig. 5.4). This MSSA strain exhibited between 196–240 SNPs to Clade III Sri Lankan clone isolates and 179 SNPs to the comparator Z4294 isolate. Finally, the Irish PVL-positive MSSA comparator isolate (M190008) with the intact phage grouped with the other outgroup comparator isolates at the base of the MLT (shaded in orange in Fig. 5.4).

5.3.3 Genotypic profiling of the pubMLST isolates

5.3.3.1 Strain assignment

Analysis of the antimicrobial resistance and virulence genes associated with the pubMLST isolates revealed that these strains shared close similarities to the Sri Lankan clone isolates described in Chapter 4 of this study. The majority of Sri Lankan clone isolates (200/214; 93.5%) from Chapter 4 were classified as 'CC5-MRSA-IVc (*sed*/*sej*/*ser*+)'. Similarly, all of the pubMLST isolates (18/18) also harboured the *sed*/*sej*/*ser* enterotoxin genes (Table 5.3).

5.3.3.2 Antimicrobial resistance genes

The *blaZ* gene was detected in the majority of the pubMLST isolates (17/18; 94.4%) (Table 5.3), as with the majority of Sri Lankan clone isolates from Chapter 4 (202/214; 94.4%). The *fosB* gene and *sdrM* were also detected in all the pubMLST isolates (18/18) (Table 5.3) and in the majority of Sri Lankan clone isolates from Chapter 4 (213/214; 99.5%). The *lmrP* gene was detected in all the pubMLST isolates (Table 5.3) and in most of the Sri Lankan clone isolates from Chapter 4 (212/214; 99.1%). The majority of the PVL-positive ST5-MRSA-IVc (7/10; 70%), ST5-MRSA-IVa (4/7; 57%) and the ST5-MSSA (1/1) pubMLST isolates harboured the erythromycin resistance *erm*(C) gene (Table 5.3). This gene was also detected in 41.1% of the Sri Lankan clone isolates from

Chapter 4. Only one of the PVL-positive ST5-MRSA-IVc pubMLST isolates (1/10; 10%) carried the tetracycline resistance *tet*(K) gene (Table 5.3), comparable to Chapter 4 where only 6% of the Sri Lankan clone isolates carried this gene.

5.3.3.3 Immune evasion cluster types

IEC-type G was predominant amongst the pubMLST isolates (17/18; 94.4%) (Table 5.3), as with the Sri Lankan clone isolates from Chapter 4 (196/214; 91.6%). The remaining PVL-positive ST5-MRSA-IVa pubMLST isolate carried no IEC genes and harboured an intact, untruncated *hlb* gene.

Collectively, these findings indicate that the *in silico* PCR analysis permitted the detection of ten additional PVL-positive ST5-MRSA-IVc Sri Lankan clone isolates and eight other comparator isolates (including a ST5-MSSA strain) which all harboured an identical PVL-encoding phage remnant and similar molecular characteristics as the isolates described in Chapter 4 of this study.

5.4 Discussion

The first comprehensive report of the PVL-carrying CC5/ST5-MRSA-IVc Sri Lankan clone was provided by McTavish *et al.,* in 2019 following its detection in a Sri Lankan hospital. Chapter 3 and 4 of this study provided insights into the prevalence of this clone, illustrating its expansion across Europe, Asia and the Middle East, including its involvement in an MRSA infection outbreak within a maternity hospital in Ireland. In this chapter, a WGS-based molecular analysis was performed to characterise the PVL phage harboured by this emerging PVL-positive CA-MRSA lineage. The PVL-encoding region of Sri Lankan clone isolates was investigated against the genome of representative PVL phages commonly associated with *S. aureus* strains.

Within the Sri Lankan isolates investigated, approximately 80% of the phage genome had been deleted leaving behind a 9.6 kb PVL-encoding remnant integrated into the chromosome of the bacteria (Fig. 5.5). A search of the literature revealed no other instances where this remnant had been reported, suggesting that this feature may be a useful genetic marker for the Sri Lankan clone. The presence of the 9.6 kb remnant within the chromosome of all the Sri Lankan clone isolates investigated, including the earliest study isolate (141087, recovered in 2005), indicates that this feature was present in the early history of the PVL-positive ST5-MRSA-IVc Sri Lankan lineage. Carriage of the entire PVL prophage genome possibly imposed a fitness cost on the Sri Lankan clone or its progenitor(s), resulting in imprecise excision of the phage (Yoon *et al.,* 2019). Additionally, the stable retention of only the *pvl lukF/S-PV* genes (Fig. 5.5), while the lysogeny, morphogenesis and mobility-associated components of the phage were discarded also suggests that PVL could potentially be providing a survival advantage to the bacteria. Association of PVL-carrying *S. aureus* strains with increased virulence and enhanced transmissibility has previously been reported (Bhatta *et al.,* 2016; Jaiswal *et al.,* 2022).

Defective PVL-encoding bacteriophages with truncated tail formation genes have been described in MRSA (Kaneko and Kamio, 2004; Ma *et al.,* 2006; Wirtz *et al.,* 2009). Using electron microscopy, Kaneko and Kamio, 2004, examined a 40 kb φPVL phage isolated from a *S. aureus* strain and found no evidence of a tail structure within the phage particle, although analysis of the nucleotide sequence of the phage revealed that it

encoded tail-associated genes (Kaneko and Kamio, 2004). Furthermore, experimental attempts to infect other *S. aureus* strains with this phage particle were unsuccessful. Collectively, these findings indicated that this φPVL phage was defective. Wirtz *et al.,* 2009, also reported the presence of a defective PVL-encoding phage in the successful CA-MRSA USA300 lineage. These researchers reported that bacterial lysis and phage release allowed for successful excision and replication of the φSa3USA *hlb*-converting phage of strain USA300. However, excision/replication of the USA300-associated φSa2USA PVL-encoding phage could not be achieved. The findings of the investigation suggested that φSa2USA is a replication-defective prophage.

Interestingly, all of the PVL-positive ST5-MRSA-IVa comparator isolates which grouped into Clade II (the closest neighbour to PVL-positive ST5-MRSA-IVc Sri Lankan Clade I) and the single PVL-positive ST5-MRSA-IVa comparator isolate Z4294 located adjacent to Clade III in the cgSNP MLT also harboured the 9.6 kb phage remnant (Fig. 5.4). These findings suggest that Clade II isolates, isolate Z4294 and Sri Lankan clone Clade I/III all emerged from a PVL-positive common ancestor harbouring the 9.6 kb phage remnant, very likely a PVL-positive CC5/ST5-MSSA (Earls *et al.,* 2021). This suggestion was further supported by the detection of a PVL-positive CC5/ST5-MSSA isolate (ERR109505) in the pubMLST database by *in silico* PCR that also harboured the same 9.6 kb phage remnant. Isolate ERR109505 also grouped adjacent to Sri Lankan Clade III isolates in the cgSNP MLT (Fig. 5.4). The complete absence of SCC*mec* element sequences in this pubMLST MSSA isolate and its close phylogenetic proximity to Sri Lankan clone isolates also supports the probability of a PVL-positive MSSA progenitor giving rise to the Sri Lankan clone. It is likely that an ancestral MSSA acquired an intact PVL phage and subsequently SCC*mec* (or SCC*mec* first, then the PVL phage) and an imprecise excision event resulted in the remnant.

As the majority of Sri Lankan clone isolates investigated in this study (219/224; 98%) grouped within Clade I, this indicates that Clade I went on to disseminate widely across different continents in comparison to Sri Lankan Clade III and PVL-positive ST5-MRSA-IVa Clade II. Furthermore, as the *in silico* PCR search of the pubMLST database resulted only in the identification of CC5/ST5 *S. aureus* strains, it is likely that this phage remnant may currently be conserved to CC5. At present, all publicly available genome databases have been exhausted in the search for relevant CC5/ST5 *S. aureus* strains for

this study. Further contact with international collaborators also yielded a limited number of isolates. It is currently common practice for researchers to upload isolate WGS sequences into public databases without also providing complete metadata describing attributes of the samples (country/year of recovery, isolate type etc.). Similarly, the paucity of WGS data on MSSA isolates is also a significant hindrance to epidemiological investigations such as the one carried out in this study. During WGS-based investigations, MSSA isolates are often disregarded and only a limited number of these isolates are sequenced. The provision of complete metadata and increased sequencing of MSSA strains would contribute significantly to mitigating these limitations.

To definitively trace the exact origin, evolutionary pathway and emergence of Sri Lankan Clade I and the phage remnant, further investigations with older CC5/ST5 MRSA and MSSA strains harbouring the same 9.6 kb phage are required. With increasing levels of PVL-positive CA-MRSA strains being reported worldwide (Bhatta *et al.,* 2016; Klein *et al.,* 2020), including in Ireland (NMRSARL 2020; Shore *et al.,* 2014), understanding the origins and factors associated with the emergence of these lineages is of great importance.

Chapter 6

General Discussion

6.1 CA-MRSA in Ireland

The work presented in this thesis provides the most detailed insights to date into the diverse population of CA-MRSA currently circulating in Ireland. While the prevalence and epidemiology of HA-MRSA in Ireland has been extensively investigated, data on CA-MRSA epidemiology is scarce and incomplete. Unlike many other countries, there are currently no formal surveillance systems in place to monitor the prevalence and spread of CA-MRSA in Ireland. In the USA, geographical surveillance of CA-MRSA is continuously advancing. In recent years, epidemiological investigations have been enhanced with the use of geocoded electronic health record databases to identify CA-MRSA incidence rates in the community and also identify possible risk factors, including community demographics, household crowding, gender and age (Casey *et al.,* 2013; Immergluck *et al.,* 2019; Jackson *et al.,* 2016). In Japan, a nationwide surveillance study on CA-MRSA was recently undertaken involving isolates recovered from 244 healthcare facilities over a decade (Yamaguchi *et al.,* 2022). The study analysed changes in the molecular and epidemiological characteristics of CA-MRSA and accounted for region, period of isolation, patient age and gender (Yamaguchi *et al.,* 2022).

As the incidence of CA-MRSA continues to rise globally and several successful epidemic clones are reported on frequently, epidemiological surveillance on a national level becomes increasingly critical in the fight against MRSA. To address this lack of adequate data on CA-MRSA incidence in Ireland, Chapter 3 of this study investigated the population structure and diversity of CA-MRSA in Irish nosocomial settings, including the association of numerous distinct PVL-positive CA-MRSA lineages with infection outbreaks. The findings presented in Chapter 3 revealed that the overall genetic diversity and distribution of CA-MRSA in Ireland is high. Over the 11-year study period (2011–2022) where CA-MRSA isolates were recovered primarily from maternity patients without traditional risk factors, 32 different STs and 91 different *spa* types were identified. These findings reflect the increasing global prevalence of CA-MRSA. The diversity observed also highlights the considerable threat posed by MRSA in Ireland. According to Health Protection Surveillance Centre (HPSC) correspondence with the Coombe Women's and Infants University Hospital, Ireland in 2010, a three-year survey of CA-MRSA colonisation in maternity patients revealed a prevalence of 1.6% (Department of Health, Ireland, 2013). A similar 2-year study of obstetrics patients in a Canadian hospital between 2008 and 2010 found MRSA carriage to be 0.34% among this

cohort (Wang *et al.,* 2018). Another study carried out in a Danish maternity hospital in 2015 found an even lower prevalence of 0.11% (Holm *et al.,* 2021).

According to the epidemiological work presented in Chapter 3, ST5 is the dominant CA-MRSA in Ireland, accounting for 22.7% of all CA-MRSA isolates recovered. Interestingly, ST5 also accounted for a significant proportion of all PVL-positive isolates recovered in this study (19.7%), and was associated with an outbreak spanning 15-months in an Irish maternity hospital. The findings presented in Chapters 4 and 5 further expanded on the emergence of the PVL-positive ST5-MRSA-IVc lineage responsible for this outbreak and described the global dissemination of this clone. With the changing epidemiology of MRSA, ST5 is rapidly becoming a prevalent CA-MRSA worldwide. Outbreaks of PVL-positive ST5 CA-MRSA are now common, several having occurred in NICUs, maternity units and in paediatric care units (Broderick *et al.,* 2021; Madigan *et al.,* 2018; Rokney *et al.,* 2017; Sola *et al.,* 2012). This predominance of ST5 warrants rigorous surveillance and immediate investigation of all ST5 CA-MRSA strains emerging in hospitals and in the community.

6.2 Transmission dynamics of CA-MRSA in Ireland

The results presented in this thesis are helpful in understanding the numerous reservoirs aiding the spread of CA-MRSA in Ireland specifically. Currently, national IPC measures aimed at reducing transmission appear to rely heavily on procedures developed by other countries. For example, a proportion of the national clinical guidelines provided by the Health Protection Surveillance Centre (HPSC) for reporting CA-MRSA transmission are based on strategies in place in the UK (Department of Health, Ireland, 2013). These recommendations may not be entirely relevant to Ireland, as CA-MRSA risk factors and transmission dynamics are known to vary depending on geographical locations, socioeconomic factors and sociodemographic variables (Hasanpour *et al.,* 2023; King *et al.,* 2022). A comparison between the UK and Ireland indicates significant differences in the rates of urbanisation and in the population living below the poverty line (CSO, 2022a; Francis-Devine, 2023; Government Office for Science UK, 2021). This suggests that risk factors such as household overcrowding and lower socioeconomic statuses may be considerably more relevant in one setting than the other. Additionally, despite the population growth rate in Ireland being estimated at 1% in 2020 compared to 0.4% in the UK, the diversity in the ethnic makeup of Ireland is not as extensive (The World Bank,

2020). This could also result in differences in MRSA epidemiology on a local level. Understanding the transmission dynamics at play in Ireland specifically will allow for the development of targeted infection control strategies. This study highlighted the importance of household transmission, patient-to-HCW/HCW-to-patient transmission and global travel in the persistence of MRSA in the community and its subsequent introduction into healthcare settings.

6.2.1 International travel and migration

In Chapter 3, the transmission of two distinct CA-MRSA strains (a PVL-positive ST1-MRSA-V+*fus*+*tirS*+*ccrA1* and a PVL-negative ST97-MRSA-V+*fus*) among several members of the same family was revealed. Recent travel history recorded for some members of this family indicated that they had travelled to the Middle East, suggesting possible importation of these strains into Ireland and subsequent spread within the family. Similar isolates have been recovered in the Middle East and in people who had previously travelled to and from Middle Eastern countries (Monecke *et al.,* 2012). The likelihood of zoonotic transmission of this MDR lineage into humans from livestock or domestic animals has also been extensively reported (Feltrin *et al.,* 2015; Monecke *et al.,* 2011; Monecke *et al.,* 2012). Similarly, the investigation of the PVL-positive ST5-MRSA-IVc Sri Lankan clone described in Chapter 4 identified Irish patients with recent travel to Sri Lanka and Turkey.

Increased immigration into Ireland in recent years as a result of escalating political and economic instability in other parts of the world could also be a contributing factor to the prevalence of CA-MRSA in the community. According to the Central Statistics Office (CSO), there was a 15-year high in immigration into Ireland in 2022 (CSO, 2022b). Additionally, following COVID-19 lockdowns and restrictions, travel into Europe has rebounded strongly, with tourism expected to fully return to pre-pandemic levels in 2023 (UNWTO, 2023). It has also been suggested that the COVID-19 pandemic exacerbated the global AMR problem and shifted focus away from antimicrobial stewardship in an attempt to control the spread of the virus (Elmahi *et al.,* 2022). Studies reported increased incidences of antimicrobial-resistant infections during the pandemic, increased prevalence of MDR pathogens and higher use/misuse of antibiotics, antivirals and antimalarial agents (Elmahi *et al.,* 2022; Khaznadar *et al.,* 2023). Collectively, this could all have contributed towards the increasing prevalence of CA-MRSA worldwide and

could also aid the displacement of predominant endemic clones by novel strains with increased transmissibility. Although screening patients based on international travel and migration would pose a substantial financial burden on the healthcare sector, improved documentation of patient travel history and family background would aid retrospective studies when attempting to trace strain transmission.

6.2.2 Household transmission

As described in Section 6.2.1 above, the results presented in Chapter 3 revealed intra-familial transmission of CA-MRSA in Ireland. Members of two separate families associated with hospital H2 had previously received treatment for either a serious MRSA infection or a SSTI. This led to screening of other family members within the same household to detect colonisation. Brennan *et al.*, 2012, also recently reported another instance where screening of family members led to detection of CA-MRSA household transmission in Ireland. These researchers identified a HCW who had transmitted the ST772-MRSA-V Bengal Bay clone to her child, as well as to other NICU staff and patients. Household environments serve as a key reservoir for transmission of CA-MRSA, acting as an intermediate source for transmission to other household contacts (Davis *et al.,* 2012; Scott *et al.,* 2008). The index first colonises surfaces within the home, which then maintains the transmission cycle of MRSA. This also plays a role in recolonisation of patients who had previously undergone antibiotic treatment or decolonisation (Davis *et al.,* 2012). Survival of MRSA within the home environment is suggested to be dependent on fomites such as dust composition, surface material as well as temperature, humidity and strain type, which all highlight the importance of good hygiene practice to reduce transmission (Davis *et al.,* 2012). Focusing primarily on patient decolonisation is not sufficient in preventing the spread of MRSA in the community, particularly with growing concerns regarding chlorhexidine and mupirocin resistance (Hardy *et al.,* 2018). Household environmental decontamination should be considered as an important measure to control household transmissions, specifically in the homes of patients who are persistent carriers.

6.2.3 Healthcare workers

The involvement of HCWs in the transmission of CA-MRSA within healthcare settings was also highlighted in this study. Among the outbreaks described in Chapter 3, a staff member within the maternity unit of a regional Irish hospital was associated with a ST88-

MRSA-V outbreak. Recent studies by Kinnevey *et al.*, in 2021 and 2022, undertaken in a large-acute Dublin-based hospital also extensively investigated the role played by HCWs in the transmission of MRSA/MSSA in non-outbreak settings. Numerous transmission events involving HCW-to-patient, HCW-to-HCW and environmental contamination due to shedding of MSSA/MRSA isolates by HCWs and patients were evident in these studies. Although patient-to-patient transmission events were also observed, the researchers concluded that only a minority of these *S. aureus* hospital acquisitions were as a result of patient-to-patient transmission. Additionally, a recent study by Sassmannshausen *et al.* 2016, into the prevalence of MRSA amongst different HCWs revealed higher prevalence in nurses than in medical doctors, possibly as a result of closer, more frequent contact between nurses and patients.

Despite approximately one-third of the general population being carriers of *S. aureus* (Sakr *et al.,* 2018), screening of HCWs for MSSA is not normally undertaken (Department of Health, Ireland, 2013). In regard to MRSA, the current national IPC guidelines in Ireland only recommend screening HCWs upon identification of a possible link with an infection outbreak cluster (Department of Health, Ireland, 2013). Routine investigation of environmental MRSA is also not common practice in most countries including Ireland (Department of Health, Ireland, 2013). Good IPC practice necessitates monitoring the effectiveness of cleaning and decontamination practices. Improved hygiene practices and more thorough and effective environmental decontamination strategies should be considered for healthcare settings, particularly during patient transfer. Secondly, periodic staff screening for MSSA and MRSA should also be considered, particularly for nurses and healthcare assistants, irrespective of associations with known outbreaks. The primary barriers currently affecting the implementation of periodic staff screening include cost and a general reluctance among HCWs to screening as positive results may result in exclusion from work or feelings of stigma. However, the risk of *S. aureus* transmission from HCWs to vulnerable patients and subsequent infection greatly outweigh these concerns. In the Netherlands where active surveillance screening on HCWs is regularly performed, MRSA-positive staff are often assigned to non-patient contact work areas, offered decolonisation therapy and promptly returned to their regular roles following successful decolonisation (Lekkerkerk *et al.,* 2017; Souverein *et al.,* 2016). This approach contributes towards the lower MRSA colonisation rates in the Dutch population compared with other nations (Souverein *et al.,* 2016).

6.2.4 Key improvements to IPC measures to limit transmission

As evidenced by recent epidemiological investigations carried out in Ireland and from the findings presented in this study (Brennan *et al.,* 2012; Broderick *et al.,* 2021; Earls *et al.,* 2018; Earls *et al.,* 2017; Earls *et al.,* 2019; Kinnevey *et al.,* 2021; Kinnevey *et al.,* 2022; Shore *et al.,* 2014), IPC resources currently allocated towards managing the complex public health challenges presented by MRSA are inadequate. In countries with low-intermediate MRSA prevalence rates, such as the Netherlands, risk assessments are carried out following hospital admission to identify MRSA risk groups (Westgeest *et al.,* 2022). This is followed by deployment of a 'search and destroy' policy whereby patients who are deemed high-risk are screened and isolated (Westgeest *et al.,* 2022). This approach has been crucial in maintaining low MRSA colonisation and infection rates. In countries with endemic MRSA prevalence, universal patient screening upon admission has been proposed as a potential tool in controlling the spread of MRSA, although the cost-benefit of this approach is subject to debate (Borg *et al.,* 2021; Roth *et al.,* 2016). In the UK, the effectiveness of mandatory admission screening has been heavily scrutinised (Care, 2014; Deeny *et al.,* 2013). Targeted screening followed by decolonisation has been suggested as a more effective approach in this setting (Deeny *et al.,* 2013), although non-compliance with universal screening could potentially have hindered the success of this approach. According to the Department of Health and Social Care, a policy recommending mandatory MRSA screening in the UK was followed in less than two-thirds of admissions (Department of Health, UK, 2014).

The application of either universal screening or upgrades to current targeted screening practices in Ireland would require improvements to existing hospital designs. Severe overcrowding in Irish hospitals has resulted in over-reliance on multi-bed wards, making it difficult to adequately isolate patients in single-bed rooms (Irish Nurses and Midwives Organisation, 2022). Additionally, hospital overcrowding also results in bed shortages and high bed occupancy rates. With high demands for hospital beds, HCWs and cleaning staff have limited time to effectively clean and decontaminate patient care equipment and shared bathroom facilities between patients, which may result in inadequate decontamination. Contaminated hospital environments are well recognised reservoirs for transmission of pathogens, even after the colonised/infected patient has vacated the environment (Boyce, 2007). Despite increased over-capacity in Irish hospitals, only 162 additional inpatient beds are due to become available nation-wide in 2023 (The Irish

Times, 2023). The rapidly growing population in Ireland will also further contribute to the current pressure on healthcare services, including demands for more hospital beds. According to national Central Statistics Office data, the population of Ireland increased by 8% between 2016 and 2022, and now exceeds the 5 million threshold for the first time since 1851 (CSO, 2022c). Additional government investment into the healthcare system is crucial in lowering MRSA transmission rates in Ireland. A variety of approaches should be considered, including (i) the provision of more single-occupancy rooms in hospitals, (ii) cohorting of patients according to MRSA colonisation status when isolation rooms are unavailable and (iii) designating staff to caring for MRSA patients to avoid further transmission to non-colonised patients.

6.3 Antimicrobial resistance in Ireland

The findings presented in this study also reflect the ongoing antimicrobial resistance crisis, which is considered one of the top ten global public health threats according to the World Health Organisation (WHO) (Pariente *et al.,* 2022). Traditionally, resistance to multiple classes of clinically relevant antibiotics is not a common characteristic of CA-MRSA, but is increasingly being reported among PVL-positive CA-MRSA strains worldwide (Udo, 2013). In Chapter 3 of this study where outbreak-associated CA-MRSA strains were investigated, MDR was detected in 37% of these strains. Most notably, the PVL-positive ST1-MRSA-V and PVL-negative ST97-MRSA-V intra-familial strains exhibited phenotypic resistance to fusidic acid and also carried the *fus* gene. Although systemic use of fusidic acid has significantly reduced in Ireland, it is still readily prescribed as a topical treatment for SSTIs (Earls *et al.,* 2017). Additionally, importation of SCC*fus* carrying MRSA strains from countries where inappropriate use of topical antibiotics is common could also be occurring. Numerous studies have concluded that the widespread use of topical antimicrobial agents such as fusidic acid and mupirocin often results in increased bacterial resistance and reduced susceptibility to these agents (Carter *et al.,* 2018; Nong *et al.,* 2021). Prolonged exposure to these topical agents could also potentially be contributing to the emergence of MDR CA-MRSA strains, as *fusC* or *mupA*-harbouring *S. aureus* strains can co-select for other antimicrobial resistance genes such as *mecA*, *blaZ* and *qacA* (Carter *et al.,* 2018). Currently, IPC protocols recommend focus should be placed on treating the underlying skin condition first before decolonisation can be considered. To reduce the introduction of CA-MRSA into healthcare settings from the community, patients with skin infections should be isolated

and offered decolonisation treatments following admittance (Department of Health, Ireland, 2013).

According to HPSC reports, outpatient use of antibiotics is mid-to-high in Ireland in comparison with other reporting European countries (HPSC, 2023). Interestingly, there is also high seasonal fluctuations in antibiotic use in Ireland, with winter use appearing to be significantly higher than summer use (Fig. 6.1a) (HPSC, 2023). This indicates the possible misuse of antibiotics as a treatment option for seasonal influenza and other viral respiratory infections. The use of broad-spectrum antibiotics such as macrolides and fluoroquinolones have remained stable in recent years in Ireland, and is relatively high in comparison to other European countries, despite associations with MRSA selection/resistance (HPSC, 2023; Department of Health, Ireland, 2013).

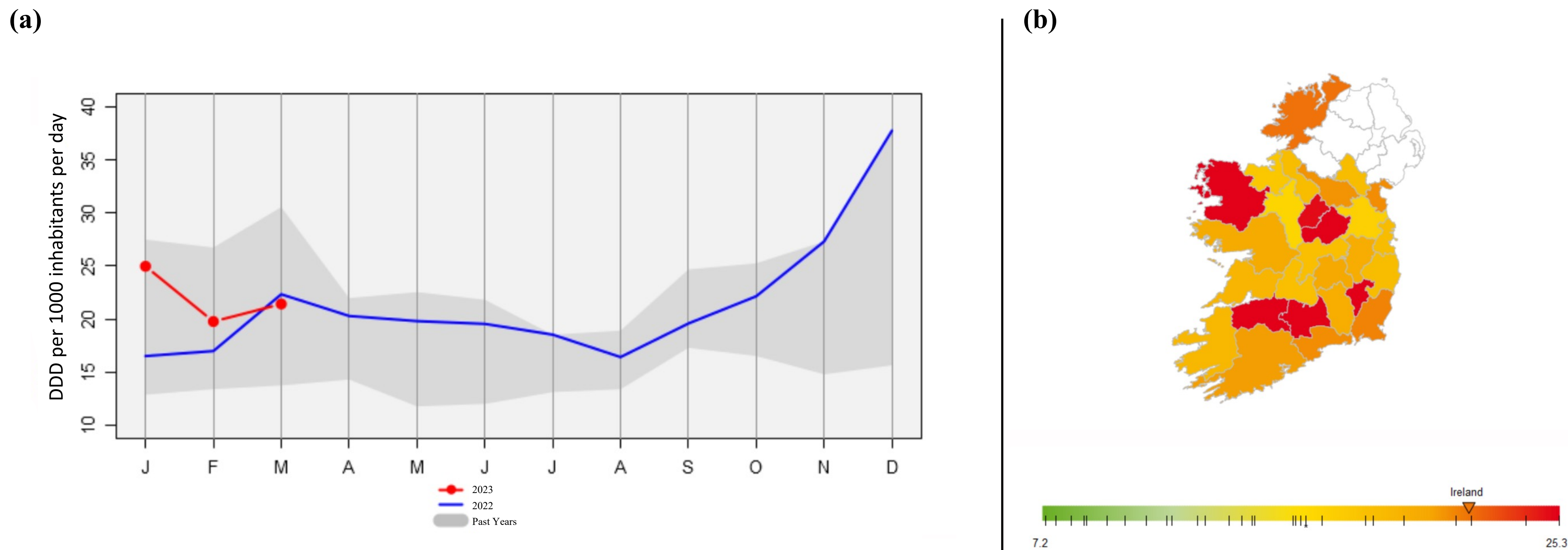


Figure 6.1. Antibiotic consumption in the community in Ireland. **(a)** Total outpatient antibiotic consumption in Ireland by month from January to December expressed in Defined Daily Doses (DDD) per 1000 inhabitants per day. Seasonal fluctuation showing latest data for year 2023 in red, 2022 in blue and past years in grey. **(b)** Regional variations in rates of antibiotic consumption in the community in Ireland. Data for latest year provisional to 2023Q1. The level of antibiotic consumption for each country in the European Centre for Disease Prevention and Control (ECDC) map is indicated with a black line (|). Rate for Ireland when ECDC map was last published is indicated with an asterisk (*). Current rate for Ireland is indicated by the triangle. Adapted from Health Protection Surveillance Centre - Public MicroB Report Viewer (HPSC, 2023).

Earls *et al.,* 2017 recently reported on the transmission of the MDR European CC1-MRSA-IV clone within and between hospitals in Ireland and the involvement of HCWs in these transmission events. This CA-MRSA clone encodes resistance to MLS_B (macrolide, lincosamide and streptogramin B) compounds, aminoglycoside-streptothricin antibiotics (*aadE-sat-aphA3*), tetracycline [*tet*(K)] and β-lactams (*blaZ*) (Earls *et al.,* 2017).

There is also wide regional variations at county level in the use of antibiotics in Ireland (Fig. 6.1b) (HPSC, 2023). This suggests that awareness and education regarding misuse and overuse may require a targeted approach. Additionally, guidelines for prescribing antibiotics need to be reviewed and further standardised. Considering that the annual expenditure for medicines in Ireland currently exceeds €2.5b (approximately 14% of the total national health budget and ~7% of GDP) (Global Legal Insights, 2022), reducing inappropriate use of antibiotics could contribute significantly towards tackling the problem of AMR and also help to alleviate some of the economic burden on the healthcare sector.

In livestock settings, lineages such as CC398, CC130 and CC1/t127 identified in colonised farm animals are considered emerging reservoirs of MRSA for transmission to humans (Elstrøm *et al.,* 2019). These strains frequently harbour a wide variety of AMR genes, possibly as a direct consequence of the extensive use of antibiotics in agriculture (Cuny *et al.,* 2015). Livestock and livestock-associated *S. aureus* strains are also frequently exposed to antimicrobial agents and heavy metals which are naturally present in the environment or introduced through pollution by farm disinfectants and preservatives (Cuny *et al.,* 2015; Dweba *et al.,* 2018). This exposure sometimes results in co-selection of AMR genes with heavy metal resistance genes by *S. aureus* strains (Dweba *et al.,* 2018). For example, the classic livestock-associated CC398-MRSA-V lineage typically exhibits high-level resistance to β-lactams, tetracyclines, macrolides, aminoglycosides, cadmium and zinc (Matuszewska *et al.,* 2022; Sharma *et al.,* 2016). Human-adapted variants of CC398 more commonly only exhibit resistance to macrolides (Bouiller *et al.,* 2022; Matuszewska *et al.,* 2022). Similarly, the CC130-MRSA lineage commonly found in dairy cattle carrying the SCC*mec*XI–*blaZ*/*mec*C element, *tet* efflux and arsenic resistance operon genes (*arsB*/*arsC*) has also been recovered from humans, including in Irish patients (Kinnevey *et al.,* 2014; Shore *et al.,* 2011). Interestingly, this

lineage has also been associated with European hedgehog populations, which are now regarded as long-standing reservoirs of *mec*C-MRSA, even predating the advent of the antibiotic era (Larsen *et al.,* 2022).

Fortunately, significant progress has been made in reducing the overall use of antibiotics in livestock as a result of effective EU legislation. This has resulted in a 47% reduction in annual sales of antibiotics for animal use in the EU/EEA, Switzerland and UK in the last decade (FEFAC, 2022). New regulations have also placed a ban on routine prophylactic antibiotic use in livestock and recommends preventative use for exceptional cases only (Simjee and Ippolito, 2022). According to the World Organisation for Animal Health (WOAH), global antibiotic use in animals has also reduced by 27% in the last three years (Animal Health Europe, 2022). With increasing reports of zoonotic transmissions of MRSA to humans over the last decade (Anjum *et al.,* 2019; Butaye *et al.,* 2016; Elstrøm *et al.,* 2019), these are much needed initiatives.

6.4 Superiority of WGS-based typing methods for investigating *S. aureus*

The current study demonstrated the high resolution and unparalleled discriminatory abilities offered by WGS over conventional molecular typing methods. In Chapters 3, 4 and 5, various WGS-based typing tools were employed for a combined epidemiological and phylogenetic approach to (i) investigate CA-MRSA outbreaks and transmission within and outside healthcare settings, (ii) describe the CA-MRSA population structure in Ireland and (iii) elucidate the widespread dissemination of a novel emerging clone internationally.

In the outbreak studies presented in Chapter 3, the phenotypic antimicrobial susceptibility profiles, *spa* types and close proximity in recovery dates (December 2017–March 2018) of t008/ST8-MRSA-IVa isolates from two separate Irish hospitals (H1 and H2) inferred high genetic similarities between these isolates, thus suggesting possible transmission between the two Dublin-based hospitals. By contrast, wgMLST-based and wgSNP-based phylogenetic analyses revealed that outbreaks within the two hospitals were caused by two genotypically distinct PVL-positive USA300 strains (Clusters CH1 and CH2 in Figs. 3.2a and 3.3a). Similarly, in the wgMLST-based and wgSNP-based presented in this Chapter, isolate M18/0106, a t723 isolate (*spa* repeat succession: 11–19–12–34–22–25) clustered closely with t008 isolates (*spa* repeat succession: 11–19–

12–21–17–34–24–34–22–25) exhibiting between 4–6 allelic differences (or 6–8 SNPs) from these isolates. Prior to the introduction of WGS-based phylogenetic tools, investigations using conventional techniques only or analysis based on *spa* types alone would prematurely have excluded this isolate from the outbreak study.

In Chapter 3, WGS-based cgMLST was also used to investigate the epidemiology of CA-MRSA in Ireland. The cgMLST MST permitted the identification of major circulating lineages, many of which possess enhanced antimicrobial resistance and virulence determinants (Fig. 3.4). A second cgMLST-based MST was also constructed to compare the emerging PVL-positive ST22-MRSA population in Ireland with epidemiologically unrelated international PVL-positive ST22 strains (Fig. 3.5b). This *in silico* analysis was facilitated by the availability of high-quality whole-genome sequences in public databases. This accessibility to large public repositories of genomes recovered from numerous disparate geographical locations across an extensive period of time highlights the powerful resource provided only by WGS.

The unrivalled sensitivity and precision offered by WGS-based technologies was demonstrated in Chapter 4 of this study. Second and third generation WGS tools were employed to produce highly accurate *de novo* assemblies, which permitted thorough investigation of the emerging novel Sri Lankan clone. A cgSNP-based MLT and cgMLST-based MST was constructed and a major clade (Clade I) of the PVL-positive ST5-MRSA-IVc Sri Lankan clone was identified (Figs. 4.4 and 4.5). The cgSNP phylogenetic investigation also permitted the identification of smaller emerging clades (Clade II and Clade III) of the PVL-positive ST5 lineage, a finding which would have been overlooked if only one molecular typing tool was employed for this analysis. Similarly, the cgMLST phylogenetic investigation permitted the detection of related isolate groups which were not easily discernible on the cgSNP MLT. This highlighted the varying applications of different WGS-based typing methods, the importance of choosing appropriate typing techniques and also the usefulness of employing multiple approaches for WGS data interpretation.

Aside from its application in high-resolution phylogenetic analyses in this study, the combination of short-read WGS data with long-read data also greatly improved the analysis of *S. aureus* isolates. The construction of higher quality genomes allow for the

detection of distinct lineage-specific genetic markers, which could potentially be relevant in understanding the divergence and evolutionary history of novel *S. aureus* clones. In Chapters 4 and 5, hybrid-assembled WGS data was used to characterise the PVL-encoding phage remnant and the *sdrE*/*bbp* gene in the emerging PVL-positive ST5-MRSA-IVc Sri Lankan clone (Figs. 4.6 and 5.5).

6.4.1 Future applications of WGS in *S. aureus* investigations

Despite advancements in sequencing technologies in recent years, adoption of WGS for routine use to guide public health surveillance and outbreak investigations in Ireland has been slow. As evidenced by the results presented in this study, hospital-based microbiology laboratories would greatly benefit from integration of WGS into routine clinical microbiology laboratories. As the primary surveillance tool, WGS would provide timely, clinically relevant information allowing for real-time investigation of transmission events and rapid management of infection outbreaks. Currently, the barriers hindering the integration of WGS into routine clinical settings include complexity of WGS data analysis as there are limited user-friendly/automated analysis software available and also lack of standardisation in regard to data analysis and quality control measures. To bridge this gap between research use of WGS and routine clinical applications, additional investments into infectious disease surveillance is needed. This would provide the resources to bring sequencing technologies in-house, particularly into acute public hospitals across the country. Further improvements could include (i) provision of training to current laboratory staff, (ii) recruitment of more researchers with specialist knowledge of WGS technologies into hospital laboratories, (iii) standardisation of WGS typing techniques to allow for interlaboratory reproducibility, particularly in terms of isolate relatedness thresholds and (iv) access to standardised software packages and user-friendly bio-informatic workflows which would allow for easier data analysis and data interpretation.

The integration of WGS into more routine clinical settings will also significantly reduce the turn-around time between patient screening and data acquisition, possibly encouraging more screening and increasing the current genome sequence data pool. From current literature, the disparities in the number of epidemiological studies carried out on MSSA relative to MRSA is clearly evident. Similarly, the availability of WGS MSSA data within public genome databases is also limited. It is suggested that MSSA strains

are often dismissed as carriage isolates, despite their associations with clinically relevant invasive infections (Zhu *et al.,* 2022). This oversight also negatively impacts parallel studies between MRSA and MSSA to determine the evolutionary history of novel emerging clones. Many of the major circulating CA-MRSA clones including the epidemic European CC80-MRSA-IV, European CC1-MRSA-IV and CC8-MRSA-IV USA300 emerged from ancestral MSSA strains (Earls *et al.,* 2021; Stegger *et al.,* 2014; Strauß *et al.,* 2017). In Chapter 5 of this study, the exact origins of the PVL-positive Sri Lankan clone could not be accurately determined as only a limited number of potential progenitor MSSA sequences were recovered following an extensive search. To gain further insights into the emergence of this clone, increased availability of MSSA genome data is required.

6.5 Concluding remarks

The emergence of CA-MRSA is a clinical and epidemiological problem which has had serious implications on global public health and infection prevention. The epidemiology of MRSA has evolved rapidly in recent years as the boundaries and distinctions between CA-MRSA and HA-MRSA blur. There is now frequent introduction of CA-MRSA into healthcare settings and reports of HA-MRSA transmission in the community, with significant overlap in the MDR profiles and virulence determinants of these emerging strains. Similarly, increasing reports of human infections caused by LA-MRSA strains and transmission of LA-MRSA between humans in the community also demonstrates the limited relevance of current epidemiological distinctions. As major reservoirs of MRSA continue to emerge and thrive outside the healthcare environment, the traditional HA-MRSA, CA-MRSA and LA-MRSA classifications may no longer be appropriate. There is a need for better, more comprehensive approaches to MRSA surveillance which will acknowledge the changing epidemiology of this pathogen. At present, the discriminatory power offered by conventional surveillance tools such as MLST and/or *spa* typing is insufficient for accurately identifying transmission routes, confirming outbreaks and investigating complex genomic characteristics. With complete integration of high resolution WGS-based technologies into routine epidemiological investigations at a local, regional and national level, the current weaknesses in existing surveillance systems and infection control policies can be addressed.

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Appendix

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Multiple distinct outbreaks of Panton—Valentine leucocidin-positive community-associated meticillin-resistant *Staphylococcus aureus* in Ireland investigated by whole-genome sequencing

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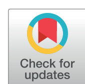

SUMMARY

Background: Panton—Valentine leucocidin (PVL)-positive community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) is increasingly associated with infection outbreaks.
Aim: To investigate multiple suspected PVL-positive CA-MRSA outbreaks using whole-genome sequencing (WGS).
Methods: Forty-six suspected outbreak-associated isolates from 36 individuals at three separate Irish hospitals (H1—H3) and from separate incidents involving separate families associated with H2 were investigated by whole-genome multi-locus sequence typing (wgMLST).
Findings: Two clusters (CH1 and CH2) consisting of 8/10 and 6/6 PVL-positive t008 ST8-MRSA-IVa isolates from H1 and H2, respectively, were identified. Within each cluster, neighbouring isolates were separated by ≤5 allelic differences; however, ≥73 allelic differences were identified between the clusters, indicating two independent outbreaks. Isolates from the H3 maternity unit formed two clusters (CH3—SCI and CH3—SCII) composed of four PVL-negative t4667 ST5-MRSA-V and 14 PVL-positive t002 ST5-MRSA-IVc isolates, respectively. Within clusters, neighbouring isolates were separated by ≤24 allelic differences, whereas both clusters were separated by 1822 allelic differences, indicating two distinct H3 outbreaks. Eight PVL-positive t127 ST1-MRSA-V+*fus* and three PVL-negative t267 ST97-MRSA-V+*fus* isolates from two distinct H2-associated families FC1 (*N* = 4) and FC2 (*N* = 7) formed three separate clusters (FC1 (t127), FC2 (t127) and FC2 (t267)). Neighbouring isolates within clusters were closely related and exhibited ≤7 allelic

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differences. Intrafamilial transmission was apparent, but the detection of ≥48 allelic differences between clusters indicated no interfamilial transmission.
Conclusion: The frequent importation of PVL-positive CA-MRSA into healthcare settings, transmission and association with outbreaks is a serious ongoing concern. WGS is a highly discriminatory, informative method for deciphering such outbreaks conclusively.
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Introduction

Community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) infections were originally defined as those occurring in otherwise healthy populations without traditional healthcare-associated MRSA (HA-MRSA) risk factors [1]. CA-MRSA infections can range from superficial skin and soft tissue infections (SSTIs) to life-threatening illnesses [2].

Several genotypic characteristics of CA-MRSA strains previously indicated an evolutionary trajectory independent to that of HA-MRSA clones, which was subsequently confirmed by whole-genome sequence (WGS)-based phylogenetic studies [1,2]. CA-MRSA lineages typically carry smaller staphylococcal chromosomal cassette elements harbouring *mec* (SCC*mec*) such as SCC*mec* types IV and V, fewer antimicrobial resistance determinants and larger arsenals of virulence factor-encoding genes than HA-MRSA. These are considered contributory factors in the ability of CA-MRSA to infect otherwise healthy individuals. The boundaries between CA-MRSA and HA-MRSA have increasingly blurred as CA-MRSA lineages have diversified and become increasingly prevalent in hospitals and other healthcare settings [1].

The expression of Panton–Valentine leucocidin (PVL) was originally considered one of the hallmark traits of CA-MRSA; however, reports of PVL-negative CA-MRSA have been increasing [3]. PVL is encoded by the *lukF-PV* and *lukS-PV* genes located in the genomes of a range of lysogenic bacteriophages. SSTIs are considered the classic presentation of PVL-positive CA-MRSA strains, although more serious infections such as necrotizing pneumonia may ensue.

Meticillin-susceptible *S. aureus* (MSSA) isolates belonging to clonal complex (CC)8 and multi-locus sequence typing (MLST) sequence type (ST) 8 emerged from Central Europe and spread to the USA about 160 years ago [4]. There it developed into the well-documented CA-MRSA clone USA300, following the acquisition of SCC*mec* IVa, the *pvl* genes, and the arginine catabolic mobile element (ACME), the latter of which is thought to enhance its ability to persist on human skin [4,5]. The USA300 clone has since achieved global spread although it has yet to become endemic outside of North America, probably due to competition by native CA-MRSA clones in these regions [4,6]. Whereas the CA-MRSA population in Europe is diverse, the prevalence of USA300 is increasing [7,8].

The PVL-positive ST5-MRSA-IV USA800 clone is well dispersed globally and predominantly associated with CA- and HA-SSTIs [9,10]. Recent WGS studies revealed a closely related PVL-positive CC5-MRSA-IVc clade of the USA800 clone (known as the Sri Lankan clade), comprising isolates from Sri Lanka, the UK, and Australia, and the association of the USA300 clone with infection outbreaks in neonatal intensive care units [10,11].

In Ireland, PVL-negative ST22-MRSA-IV remains the predominant cause of MRSA bloodstream infection (BSI), and the prevalence of PVL-positive MSSA is also low (0.8%) [12]. The proportion of PVL-positive MRSA isolates submitted to the Irish National MRSA Reference Laboratory (NMRSARL) has gradually increased since 2002 (Supplementary Figure S1) [13,14]. Between 2011 and 2017, the PVL genes were detected in an average of 9.5% of MRSA isolates sent to the NMRSARL. These PVL-positive isolates harboured SCC*mec* types IV or V and predominantly belonged to CC5 (14.7%), CC8 (31.4%), or CC30 (17.6%) [13–15]. Outbreaks caused by PVL-positive ST772-MRSA-V (known as the pandemic Bengal Bay clone) and PVL-negative ST78-MRSA-IVa and ST1-MRSA-IV [16,17] CA-MRSA lineages have been reported in Irish hospitals also. Earls *et al.* recently demonstrated the transmission of multidrug-resistant PVL-negative ST1-MRSA-IV isolates, originally considered a CA-MRSA lineage, within and between hospitals in Ireland and the involvement of healthcare workers (HCWs) in transmission events [17].

The advent of WGS has revolutionized the epidemiological investigation of microbial pathogens over the last decade. In laboratories where WGS is the most cost-effective method for highly informative molecular typing and the required bioinformatic tools are available, conventional molecular typing approaches have largely been replaced by WGS-based techniques, although isolate lineages are still described according to conventional MLST. WGS has facilitated the expansion of the traditional *S. aureus* MLST scheme based on seven loci to a core genome (cg) MLST scheme based on 1861 loci, providing a globally available, standardized, and highly discriminatory method for strain comparison and population structure investigations [18]. Strain discrimination resolution can be further enhanced using whole-genome (wg) MLST, which analyses a total of 3904 loci including the cgMLST loci, or single nucleotide variation (SNV) analysis for the investigation of infection outbreaks or transmission studies [19]. Isolates exhibiting ≤24 wgMLST or cgMLST allelic differences or ≤15 SNVs are deemed closely related and indicative of recent transmission [20].

The purpose of the present study was to use WGS to investigate the genetic relatedness of isolates recovered during distinct outbreaks caused by ST8, ST5, and ST1 PVL-positive MRSA within healthcare and community settings in Ireland.

Methods

Bacterial isolates

Forty-six MRSA isolates from 36 individuals suspected of being involved in several distinct infection outbreaks between 2011 and 2020 were submitted to the NMRSARL for routine analyses and investigated here (Table I). Thirty-five isolates were from patients or HCWs who had previously attended or worked at one of three Irish hospitals (H1–H3). Eleven isolates were recovered from members of two separate families, each of which had at

Table I
Community-associated meticillin-resistant *Staphylococcus aureus* isolates investigated in the present study

Source[a]	Individuals (*N*)	No. of isolates investigated (*N*)					
		Isolates (*N*)	*spa*-ST-SCC*mec* (*N*)	Antibiotic resistance	AR genes	PVL	IEC type
CC8-MRSA							
H1	8	10	t008-ST8-IVa (9)	Ap, Kn, Nm, Er, Cp (2)	*aphA3*, *blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF*, *msr*(A) (2)	Pos (10)	B (8)
			t723-ST8-IVa (1)	Ap, Cp (8)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mprF* (8)		C (1)
							E (1)
H2	2	6	t008-ST8-IVa (6)	Ap, Cp (6)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mprF* (6)	Pos (6)	B (6)
CRFs	10	10	t008-ST8-IVa (10)	Kn, Nm, Ap, Er, Cp (5)	*aphA3*, *blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF* (2)	Pos (10)	B (9)
				Kn, Nm, Ap, Er, Cp, Mp (1)	*aphA3*, *blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF*, *msr*(A) (3)		E (1)
				Ap, Cp (1)	*aphA3*, *blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF*, *msr*(A), *qacC* (1)		
				Ap, Er (1)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF* (1)		
				Ap, Er, Cp (2)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mph*(C), *mprF*, *msr*(A) (3)		
CC5-MRSA							
H3	18	19	t002-ST5-IVc (15)	Ap (14)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mprF* (15)	Pos (18)	B (2)
			t4667-ST5-V (4)	Ap, Cp (1)	*aacA-aphD*, *blaZ*, *mecA*, *mprF* (4)	Neg (1)	G (16)
				Gn, Kn, Tb, Ap, Cp (4)			F (1)
CRFs	7	7	t002-ST5-IVc (6)	ND (2)	*blaZ*, *fosB*, *lmrP*, *mecA*, *mprF* (3)	Pos (7)	G (7)
			t002-ST149-IVc (1)	Ap, Er (3)	*blaZ*, *erm*(C), *fosB*, *lmrP*, *mecA*, *mprF* (3)		
				Ap (1)	*blaZ*, *fosB*, *fusC*, *lmrP*, *mecA*, *mprF* (1)		
				Ap, Fd (1)			
CC1- and CC97-MRSA							
H2-FC1	3	4	t127-ST1-V+*fus*+*tir*+*ccr*A1 (4)	Gn, Kn, Nm, Tb, Ap, Fd, Te (4)	*aacA-aphD*, *aphA3*, *blaZ*, *fusC*, *mecA*, *mprF* (4)	Pos (4)	D (4)
H2-FC2	5	7	t127-ST1-V+*fus*+*tir*+*ccr*A1 (4)	Gn, Kn, Tb, Ap, Er, Fd (4)	*aacA-aphD*, *erm*(C), *fusC*, *mecA*, *mprF* (4)	Pos (4)	D (4)
			t267-ST97-V+*fus* (3)	Gn, Kn, Tb, Ap, Fd (3)	*aacA-aphD*, *blaZ*, *fusC*, *lmrP*, *mecA*, *mprF* (3)	Neg (3)	E (3)
CRFs	10	10	t127-ST1-V+*fus*+*tir*+*ccr*A1 (4)	Gn, Kn, Nm, Tb, Ap, Fd, Te (1)	*aacA-aphD*, *blaZ*, *lmrP*, *mecA*, *mprF* (2)	Pos (7)	D (1)
			t267-ST97-V+*fus* (3)	Ap, Er, Fd (1)	*aacA-aphD*, *fusC*, *lmrP*, *mecA*, *mprF* (1)	Neg (3)	E (3)
			t127-ST1-IV+*fus*+*tir*+*ccr*A1 (1)	Gn, Ak, Kn, Nm, Tb, Fd (1)	*aacA-aphD*, *aphA3*, *blaZ*, *erm*(C), *fusC*, *mecA*, *mprF* (1)		G (6)
			t127-ST859-V (1)	Ap, Te, Tp (1)	*aacA-aphD*, *aphA3*, *blaZ*, *fusC*, *lmrP*, *mecA*, *mprF* (1)		
			t127-ST1-IV (1)	Kn, Nm, Tb, Ap, Te, Tp (1)	*aacA-aphD*, *blaZ*, *lmrP*, *mecA*, *mprF*, *tet*(K) (1)		
				ND (5)	*erm*(C), *fusC*, *lmrP*, *mecA*, *mprF* (1)		
					aacA-aphD, *blaZ*, *fusC*, *lmrP*, *mecA* (1)		
					blaZ, *lmrP*, *mecA*, *mprF*, *tet*(K) (1)		
					aadD, *blaZ*, *lmrP*, *lnuA*, *mecA*, *mprF*, *tet*(K) (1)		

spa, staphylococcal protein A type; ST, sequence type; SCC*mec*, staphylococcal chromosomal cassette harbouring *mec*; AR, antimicrobial resistance; PVL, Panton–Valentine leucocidin; ND, not determined; Pos, positive; Neg, negative; H, hospital; FC, family cluster; CRFs, comparator reference isolates.

The susceptibility of the MRSA isolates to a panel of 15 antimicrobial agents including amikacin (Ak), ampicillin (Ap), chloramphenicol (Cl), ciprofloxacin (Cp), erythromycin (Er), fusidic acid (Fd), gentamicin (Gn), kanamycin (Kn), mupirocin (Mp), neomycin (Nm), rifampicin (Rf), tetracycline (Te), tobramycin (Tb), trimethoprim (Tp), and vancomycin (Vn) was determined by disc diffusion according to the Committee of Antimicrobial Susceptibility Testing methodology and interpretive criteria [22,23]. The genes commonly associated with each resistance phenotype and detected from the WGS data are listed in Supplementary Table S2.

[a] Isolates were recovered from patients with admission histories to hospitals H1–H3. Isolates associated with H3 were recovered from patients following discharge. Isolates recovered from each family cluster (FC) were referred to as H2 isolates, as two members from each FC presented at the A&E department of, or were admitted to, H2. Epidemiologically unrelated comparator isolates were submitted to the national MRSA reference laboratory from distinct hospitals or from internationally based researchers and were included as representative strains for comparative purposes only.

least one member who had attended the emergency department or who was hospitalized in H2 during 2011, 2012, or 2018 due to an MRSA infection (Supplementary Table S1).

Twenty MRSA isolates submitted to the NMRSARL were included as comparator reference isolates (CRFs) including PVL-positive MRSA identified as *spa* types t002, t008, and t127 recovered between 2014 and 2019 from community general practice clinics, regional Irish hospitals, or Dublin-based teaching hospitals other than those included in the present study (Supplementary Table S1).

Seven international CRFs were selected following comparison of the DNA microarray profiling patterns (see below) of isolates recovered from each H2-associated family cluster (FC) to an *S. aureus* DNA microarray profile database. This database comprises about 25,000 microarray profiles of human and animal strains recovered worldwide [21]. The seven CRFs selected exhibited highly similar array patterns to those of the FC isolates and consisted of two PVL-positive ST5 MRSA from humans, two PVL-positive ST1 MRSA from human ($N = 1$) and bovine ($N = 1$) hosts, and three PVL-negative ST97 MRSA from humans ($N = 2$) and poultry meat ($N = 1$) in three countries in the Middle East (Supplementary Table S1).

Identification, molecular characterization and antimicrobial susceptibility testing

Isolates were confirmed as *S. aureus* using the tube coagulase test and as MRSA using 30 μg cefoxitin discs (Oxoid Ltd, Basingstoke, UK). Isolates underwent antimicrobial susceptibility testing against 15 antimicrobial agents (Supplementary Table S2) as described previously [22,23]. The PVL-encoding *lukF-PV* and *lukS-PV* genes were detected by PCR, *spa* typing was performed as previously described and DNA microarray profiling was performed using the *S. aureus* Genotyping Kit 2.0 (Abbott (Alere Technologies GmbH), Jena, Germany) according to the manufacturer's instructions [24–26]. Isolate STs and SCC*mec* types were inferred based on *spa* types and/or DNA microarray profiles prior to WGS-based confirmation.

Passaged isolates

To investigate the genomic stability of each lineage investigated in this study, serial passaging of representative MRSA (CC1:M18/0051, CC5:M18/1033, CC8:M18/0227, and CC97:M18/0578) was undertaken. Isolates were reactivated from storage at −80°C on Microbank cryogenic bead vials (Pro-Lab Diagnostics, Birkenhead, UK) and single colony subcultured on fresh Columbia blood agar plates every 24 h for a total of 10 days. On days 2, 4, 8, and 10, a single colony was randomly selected and subjected to WGS.

Whole-genome sequencing

WGS was carried out on all isolates investigated using DNA extracted as described previously [25]. Libraries were prepared using the Nextera DNA Flex Library Preparation kit (Illumina, Eindhoven, the Netherlands) according to the manufacturer's instructions. Prepared libraries were subjected to paired-end sequencing using the MiSeq instrument (Illumina) using the 500-cycle MiSeq Reagent kit v2 (Illumina). Libraries were scaled to yield a minimum of ×70 coverage per isolate and the quality of each sequencing run was determined according to cluster density and Q30 values according to the manufacturer's instructions. All read datasets are available in the NCBI Sequence Read Archive as BioProject PRJNA638834.

Bioinformatic analyses

The BioNumerics software package v7.7 and incorporated SPAdes assembly software v3.7.1 (Applied Maths, Sint-Martens-Latem, Belgium) was used to perform wgMLST, pairwise SNV analyses, and to construct minimum spanning trees (MSTs) as previously described [25]. Both assembly-based and assembly-free methods were used to detect and identify MLST alleles as described previously [25]. Sequence reads were also assembled using the VELVET assembler incorporated within SeqSphere+ software version 7 (Ridom GmbH, Münster, Germany). Previously identified *spa* types and inferred STs were confirmed based on these assemblies using the web-based SCC*mec*Finder tool (https://cge.cbs.dtu.dk/services/SCCmecFinder/) [27]. The resistance and virulence task templates within SeqSphere+ were used to identify antimicrobial resistance genes and virulence factor genes, respectively.

Results

Investigation of ST8-MRSA-IV outbreaks at hospitals H1 and H2

Ten isolates recovered from eight patients at the outpatient ($N = 3$), gynaecology ($N = 1$), emergency department ($N = 1$), and neonatal intensive care unit ($N = 5$) of a maternity hospital in Dublin (H1) during a four-month period spanning 2017 and 2018 were investigated. All 10 isolates were identified as ACME- and PVL-positive ST8-MRSA-IVa, features characteristic of the USA300 clone (Supplementary Table S1). Six additional isolates recovered from two separate patients at a separate Dublin hospital (H2) during one week within the same period of the H1 outbreak were also identified as ACME- and PVL-positive ST8-MRSA-IVa. One H1 isolate was identified as *spa* type t723, whereas the remaining 15 H1 and H2 isolates were t008 (Table I). To investigate possible transmission events within and between hospitals H1 and H2, all isolates underwent WGS.

A wgMLST-based MST tree was constructed for all 16 isolates, the USA300 type strain FPR3757 (GenBank accession number CP000255.1) and 10 t008 PVL-positive MRSA-IV CRFs. This MST revealed two discrete clusters (CH1 (eight out of 10 H1 isolates) and CH2 (all six H2 isolates)) within which the majority of H1 and H2 isolates clustered, respectively (Figure 1a).

A separate SNV-based MST constructed for the same isolates but excluding the FPR3757 type strain also revealed the same discrete clusters (Supplementary Figure S2a). Isolates within CH1 and CH2 were differentiated from the USA300 type strain FPR3757 by 73 and 80 allelic differences, respectively (Figure 1a) and each cluster was separated by ≥127 SNVs (Supplementary Figure S2a). Isolates within CH1 were all closely related with a median of 0 (average: 1.5; range: 0–4) allelic differences or 0 (average: 1.1; range: 0–6) SNVs. The two remaining H1 isolates differed from CH1 isolates by ≥73 allelic differences (Figure 1a) and ≥120 SNVs (Supplementary Figure S2a). Isolates within CH2 were also closely related with a median of 0.5 (average: 0.5; range: 0–5) allelic

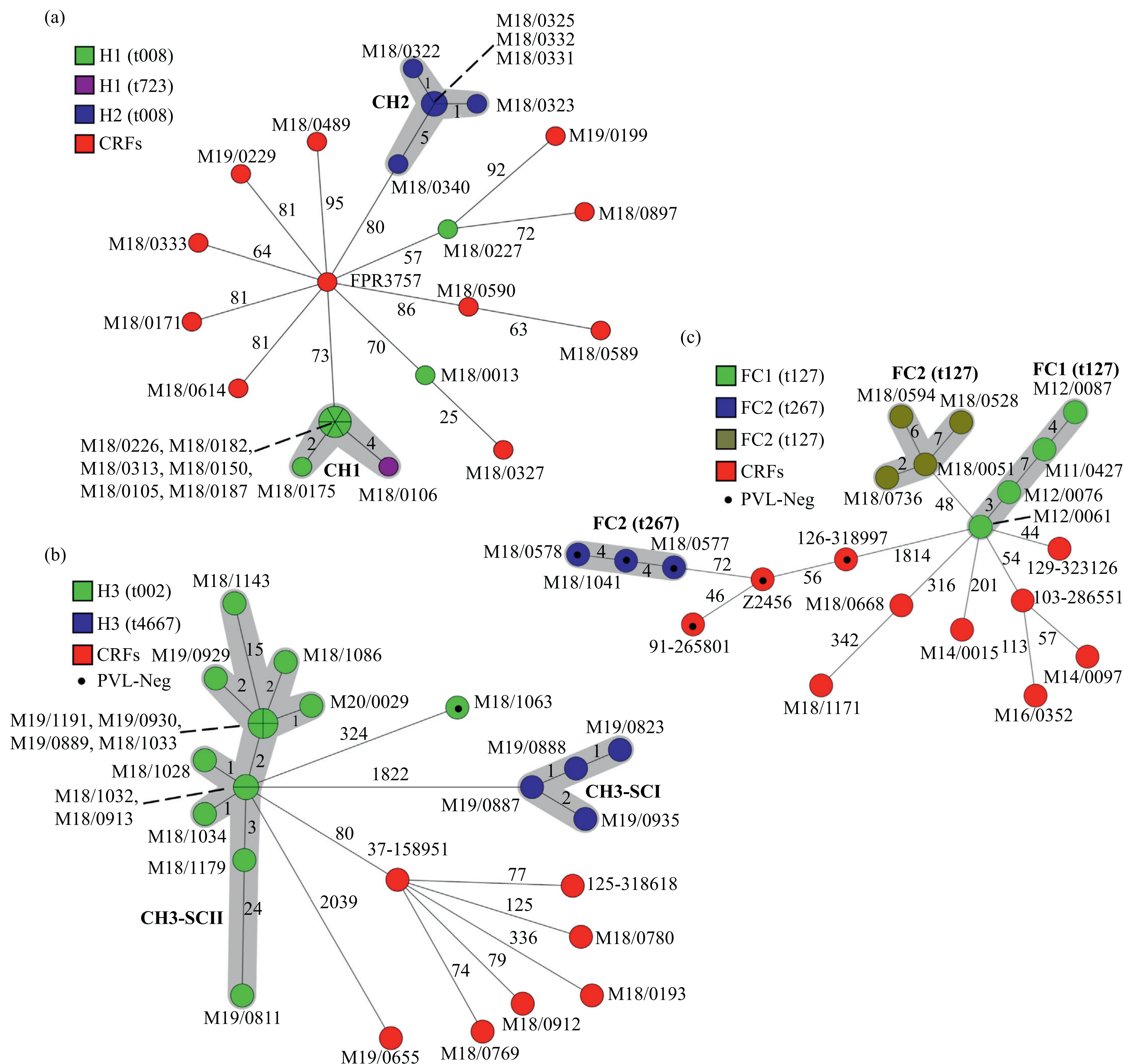

Figure 1. Minimum spanning trees (MSTs) based on whole-genome multi-locus sequencing typing (wgMLST) analysis of the 42 Panton—Valentine leucocidin (PVL)-positive and four PVL-negative MRSA isolates investigated in addition to epidemiologically unrelated but genotypically similar comparator reference isolates (CRFs). In each MST, MRSA isolates recovered from separate hospitals or families and identified as distinct *spa* types are indicated by separate colours. Genotypically similar but epidemiologically unrelated CRFs included for comparative purposes are indicated in each MST as red circles. Closely related clusters of isolates ($\leq$24 wgMLST allelic differences [20]) are outlined with grey shadowing. A black spot in the centre of each circle is used to indicate PVL-negative isolates which were included in the study as they were identified as the same *spa* types/STs as the outbreak-associated PVL-positive MRSA and were recovered in the same hospital over the relevant time periods. The PVL-negative t267 isolates were included as they were also recovered from one of the families affected by the PVL-positive t127 MRSA lineage. The numbers on each branch indicate the numbers of wgMLST allelic differences detected between neighbouring isolates. The epidemiological information for each isolate and CRF investigated is shown in Supplementary Table S1. (a) MST constructed from the t008, ST8-IVa MRSA isolates associated with hospitals 1 and 2 (H1 ($N = 10$) and H2 ($N = 6$)), CRFs ($N = 10$) and the USA300 reference strain FPR3757. The two distinct clusters, CH1 and CH2, refer to isolates recovered from hospitals H1 and H2, respectively. (b) MST constructed from the t002-ST5-IVc ($N = 15$) and t4667-ST5-V ($N = 4$) isolates associated with hospital 3 (H3) and CRFs ($N = 7$). With the exception of the PVL-negative isolate M18/1063, all t002-ST5-IVc isolates formed a distinct

differences (Figure 1a) or 0 (average: 0.8; range: 0—5) SNVs (Supplementary Figure S2a).

By contrast, the 10 ST8 CRFs and the FPR3757 reference exhibited an average of 74 allelic differences between neighbouring isolates and none clustered with CH1 or CH2 (Figure 1a). These findings indicated the occurrence of two separate outbreaks caused by genetically distinct strains, one in H1 involving the 8/10 CH1 isolates and the other in H2 involving the six CH2 isolates. Whereas all 16 isolates recovered from H1 and H2 exhibited ciprofloxacin and ampicillin resistance, two distinct phenotypic antibiotic resistance patterns were identified. By contrast, the 10 CRFs exhibited five distinct antibiotic susceptibility patterns (Table I).

Investigation of ST5-MRSA outbreak at hospital H3

Nineteen isolates from 17 separate patients and one HCW associated with the maternity unit of a regional Irish hospital (H3) recovered between 2018 and 2020 were sent to the NMRSARL for epidemiological analysis. Fifteen isolates identified as t002 ST5-MRSA-IVc (commonly referred to as USA800) were recovered over a 15-month period during 2018—20 and four t4667 ST5-MRSA-V isolates were recovered during one month in 2019 (Supplementary Table S1). Eighteen were PVL-positive and one t002 isolate was PVL-negative. These isolates were recovered from patients in the maternity ($N = 10$), special care baby ($N = 5$), outpatient ($N = 1$), emergency ($N = 1$), and paediatric ($N = 1$) units, and from an HCW ($N = 1$) and subjected to WGS to determine whether the isolates were part of a protracted outbreak or two separate outbreaks.

Both wgMLST-based (Figure 1b) and SNV-based (Supplementary Figure S2b) MSTs were constructed based on all 19 isolates, five Irish t002 CRFs, and two international PVL-positive CC5-MRSA-IV CRFs (125—318618 and 37—158951; Supplementary Table S1), which were selected on the basis of having highly similar DNA array profiles to the t002 isolates investigated here. These MSTs revealed two distinct subclusters consisting of either t4667 (subcluster CH3—SCI, $N = 4$ isolates) or t002 (subcluster CH3—SCII; $N = 14$) isolates. The PVL-negative t002 ST5-MRSA-IVc isolate from H3 (M18/1063) did not cluster with any other isolates and exhibited 324 allelic differences to the CH3—SCII subcluster (Figure 1b). All four t4667 ST5-MRSA-V isolates within CH3—SCI were closely related and exhibited a median of 1 (average: 1; range: 1—2) allelic difference (Figure 1b) and 0 (average: 0.25; range: 0—1) SNVs (Supplementary Figure S2b). These four isolates were recovered from three patients and one HCW in H3 during a one-month period in 2019 (Supplementary Table S1). All four yielded identical phenotypic antibiotic susceptibility profiles, and exhibited resistance to ≥3 classes of antibiotics (Table I). According to the wgMLST-based MST, the 14 t002 ST5-MRSA-IVc CH3—SCII isolates exhibited a median of 1 (average: 3.6; range: 0—24) allelic difference (Figure 1b). Four isolates within CH3—SCII were genetically indistinguishable (M18/1033, M19/0889, M19/0930 and M19/1191) despite being recovered from four separate patients at intervals of 11, one, and two months apart, respectively (Supplementary Table S1). Seven CRFs (Supplementary Table S1) exhibited ≥80 allelic differences to the closest relative isolate within CH3—SCII (Figure 1b). The SNV-based MST subclusters correlated with those of the wgMLST-based tree with the exception of isolate M19/0811, which was separated by its nearest CH3—SCII neighbour by 24 allelic differences (Figure 1b) or 28 SNVs (Figure S2b), respectively.

All 15 t002 isolates exhibited phenotypic ampicillin resistance and one also exhibited ciprofloxacin resistance (Table I).

Collectively, these findings revealed the occurrence of two separate outbreaks in H3, one involving ST5-MRSA-V (CH3—SCI) and the other involving ST5-MRSA-IVc (CH3—SCII).

Investigation of ST1-MRSA and ST97-MRSA isolates recovered from two separate families associated with H2

These isolates were recovered from two separate, unrelated families; referred to as FC1 and FC2. Four isolates were recovered from three FC1 members during a three-month period spanning 2011 and 2012. Of these four isolates, two were recovered from separate members who presented at the H2 emergency department with SSTIs. Two were from screening samples — one from an individual previously treated for a SSTI, and one from a third family member (Supplementary Table S1). All four isolates were identified as PVL-positive t127 ST1-MRSA and exhibited phenotypic resistance to ≥3 classes of antibiotics (Table I).

In an unrelated episode, seven MRSA isolates were recovered from five FC2 members during a seven-month period spanning 2017 and 2018. Two isolates were recovered from infections in two family members (one of whom was a H2 inpatient) and the remaining five isolates were collected from screening samples of one of these patients and three other family members (Supplementary Table S1). All seven isolates were identified as PVL-positive t127 ST1-MRSA (N=4) or PVL-negative t267 ST97-MRSA ($N = 3$) and exhibited phenotypic resistance to ≥3 classes of antibiotics (Table I).

To investigate the potential transmission of the isolates within FC2 and explore potential relatedness of the t127 isolates to those from FC1, all 11 FC1 and FC2 isolates underwent WGS. Three international PVL-negative ST97 CRFs, two international and five Irish PVL-positive ST1 CRFs were also included. Separate wgMLST and SNV-based MSTs revealed that within each distinct family cluster and *spa* type, isolates were separated from their nearest neighbour by ≤7 allelic differences or ≤8 SNVs. Isolates belonging to FC1 (t127) were separated from their nearest neighbouring isolate by a median of 4 (average: 4.6; range: 3—7) allelic differences (Figure 1c) or 1 (average: 3; range: 1—7) SNV (Supplementary Figure S2c).

Isolates belonging to FC2 (t127) were separated from their nearest neighbour by a median of 6 (average: 3.8; range: 2—7) allelic differences (Figure 1b), 5 (average: 4.7; range: 1—8)

subcluster (CH3—SCI), and all four t4667-ST5-V isolates formed a second subcluster (CH3—SCII). (c) MST constructed from the t127-ST1-V+*fus*+*tirS*+*ccrA1* ($N = 8$) and t267-ST97-V+*fus* ($N = 3$) isolates recovered from multiple members of two distinct families (FC1 ($N = 4$) and FC2 ($N = 7$)), of which each family had at least one member that either attended the emergency department of, or was admitted to, hospital 2 (H2), and 10 epidemiologically unrelated CRFs. Three distinct subclusters were apparent (FC1 (t127), FC2 (t127) and FC2 (t267)), each of which consisted of all of the isolates identified as each distinct *spa* type and from each separate family.

SNVs (Supplementary Figure S2c) and by 48 allelic differences or 52 SNVs to the FC1 (t127) isolates. Each of the t127 and t267 CRFs investigated were separated from their neighbouring isolate by ≥44 allelic differences (Figure 1c). The three *spa* type t267 isolates from FC2 were separated from each other by a median of 4 (average: 4; range: 4–4) allelic differences (Figure 1c) and 4 (average: 4; range: 4–4) SNVs (Supplementary Figure S2c). The three ST97 CRFs exhibited ≥72 allelic differences or ≥76 SNVs to the FC2 isolates.

The FC1, FC2, and CRF t127 ST1 isolates harboured genes indicative of SCC*mec* IV, V or the SCC*mec* V subtype (V+*fus*+*tirS*+*ccrA1*) (Table SI) previously identified in sporadic PVL-positive CC1 isolates [28]. The FC2 and CRF t267 ST97 isolates harboured genes indicative of SCC*mec* V+*fus* (Supplementary Table S1) previously identified in sporadic CC97 isolates [28].

Collectively, these findings indicated the transmission of distinct MRSA strains among two separate families. One t127 strain was transmitted among FC1 members, whereas two distinct t127 and t267 strains were independently transmitted among several FC2 members. Direct transmission of the t127 isolates between the two families investigated was deemed unlikely due to the identification of 48 allelic differences between the two t127 clusters (Figure 1c).

Serially passaged isolates

The wgMLST analysis of colonies randomly selected from serial passage of the ST8-MRSA-IVa isolate M18/0227, ST5-MRSA-IVc isolate M18/1033, ST1-MRSA-V+*fus*+*tirS*+*ccrA1* isolate M18/0051, and ST97-MRSA-V+*fus* isolate M18/0578 revealed a maximum of two, zero, three, and three allelic differences between each passage, respectively.

Discussion

Despite the decreasing reports of MRSA infections in Ireland in recent years, the proportion of PVL-positive MRSA has continually increased since 2002 (Supplementary Figure S1) [13,14]. The present investigation revealed the transmission of distinct PVL-positive CA-MRSA lineages within separate Irish hospitals (H1–H3). Three of these lineages (ST8-MRSA-IVa, ST5-MRSA-V, and ST5-MRSA-IVc) were associated with outbreaks in neonatal care facilities (Supplementary Table S1). Outbreaks of ST5 and ST8 CA-MRSA infections have been reported in neonatal and paediatric care units previously [11].

Multidrug resistance (resistance to ≥3 classes of clinically relevant antibiotics) is not a common characteristic of CA-MRSA, but is increasingly reported among PVL-positive MRSA populations in Europe, North and South America, Asia, and Australia [29]. In the present study, multidrug resistance was detected in 17/46 (37%) isolates, including those belonging to the ST1, ST5, ST8, and ST97 lineages (Supplementary Table S1).

The USA300 isolates were recovered from neonatal, paediatric, and adult patients in two separate hospitals. All were identified as PVL-positive t008/t723, ST8-MRSA-IVa and exhibited ampicillin and ciprofloxacin resistance (Supplementary Table S1). Despite these similarities, wgMLST revealed the presence of distinct clusters of isolates within each hospital and no evidence of transmission between the two. HCW-associated CA-MRSA transmission between hospitals in Ireland has previously been suggested, as transfer of staff between distinct healthcare facilities is common [17]. Each cluster was separated from the USA300 type strain by ≥73 allelic differences (Figure 1a) indicating separate, unrelated outbreaks. Due to the identification of t008 *spa* type and carriage of the SCC*mec* type IVa, these isolates bear the closest similarity to the clone originating in North America; however, no travel histories were available for the patients investigated [4].

Isolates identified as PVL-positive ST5-MRSA-IVc were recovered from H3-associated individuals over a 15-month period during 2018–20. These isolates were identified as *spa* types t002 and t4667, which correlated with the two distinct subclusters identified by wgMLST (Figure 1b). The t4667 isolates belonging to CH3–SCI were all recovered during the same month, harboured SCC*mec* V, exhibited multidrug resistance, and were recovered from a HCW and patients. The PVL-positive t002 isolates were recovered between 2018 and 2020, harboured SCC*mec* IVc, and exhibited ampicillin resistance (Supplementary Table S1). The low average number of allelic differences (3.4) detected among the H3 isolates compared to the average 75.7 allelic differences among CRFs is indicative of a protracted ST5 MRSA outbreak in H3.

The PVL-positive t127 ST1-MRSA isolates were from two separate families (FC1 and FC2) with links to H2; however, the FC1 t127 isolates were recovered during 2011 and 2012, whereas the FC2 t127 isolates were recovered during 2018 (Supplementary Table S1). Based on the detection of 48 allelic differences between the FC1 and FC2 t127 isolates and the fact that these isolates were recovered more than five years apart (Supplementary Table S1), it is likely that these isolates represent independent transmission networks. The ≤24 allelic differences or ≤15 SNV thresholds for inferring epidemiological relationships between *S. aureus* isolates were deemed appropriate for all lineages investigated and were supported by the available epidemiological information [20].

Similar SCC*mec* type V+*fus*+*tirS*+*ccrA1* elements were detected in all FC1 and FC2 t127 isolates and SCC*mec* V+*fus* elements were detected in the PVL-negative FC2 t267 ST97 isolates. Genes indicative of similar elements have also been identified in both PVL-positive ST1 and PVL-negative ST97 isolates recovered in the Middle East, an area where some members of FC2 had travelled, suggesting possible importation of these strains from this geographical region and subsequent intrafamilial transmission [28,30]. Previous research has shown that MRSA transmission is common in the household and can result in 67% of household contacts of an index carrier becoming colonized with the same strain [31].

The present study demonstrated the high resolution offered by WGS for investigation of outbreaks and transmission of MRSA strains both within and outside healthcare settings. For each separate investigation, distinct wgMLST- and SNV-based MSTs were congruent (Figure 1 and Supplementary Figure 2). The ST8 isolates recovered from H1 and H2 were deemed highly similar based on phenotypic antibiotic susceptibility patterns and *spa* types, suggestive of possible transmission between the two Dublin-based hospitals. However, these isolates were separated into genotypically distinct clusters by wgMLST, indicating the independent transmission of two distinct USA300 strains within each hospital and highlighting the advantage of WGS over conventional molecular typing techniques. The unparalleled discriminatory power offered by WGS also demonstrated the possibility of outbreak-associated isolates being incorrectly

excluded from outbreak investigations if based on *spa* types alone. In the present investigation, isolate M18/0106, identified as t723 (repeat succession: 11–19–12–34–22–25), exhibited between four and six allelic differences (Figure 1a), and between six and eight SNVs (Supplementary Figure S2a) to the seven t008 (repeat succession: 11–19–12–21–17–34–24–34–22–25) isolates in CH1.

As WGS becomes increasingly available to more clinical microbiology laboratories, the high resolution offered by the technology will help to inform and direct infection prevention and control strategies for outbreak management in real-time, both within healthcare facilities and/or private households. The high resolution offered by WGS can often reveal transmission events directly. This information would be highly beneficial in the implementation of routine strategies for: (i) decontamination (e.g. improved cleaning and monitoring of the most frequently outbreak-associated fomites within healthcare facilities and domestic settings); (ii) transmission risk minimization (e.g. screening of HCWs, incoming patients, and the identification of long-term carriers); and (iii) decolonization (e.g. use of topical or systemic prophylactic therapies for management of endogenous infection risk prior to admittance to healthcare facilities, which may also reduce the risk of outbreak-associated infections in healthcare settings). In addition, the WGS data can be utilized to inform approaches for antimicrobial treatments.

In the present study, two PVL-positive ST8-MRSA-IVa MRSA infection outbreaks in two Dublin-based hospitals were shown to be unrelated, a protracted PVL-positive ST5-MRSA-IVc outbreak in a Dublin-based teaching hospital was revealed and the intrafamilial transmission of PVL-positive ST1-MRSA-V+*fus*+*tirS*+*ccrA1* and PVL-negative ST97-MRSA-V+*fus* isolates in two separate families was described.

In conclusion, the prevalence of PVL-positive MRSA is continually increasing in Ireland. The frequent importation of CA-MRSA strains into healthcare settings and increasing association with HCWs and infection outbreaks is a serious concern. The application of WGS is highly advantageous in the ongoing surveillance of MRSA strains and investigation of suspected outbreaks.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2020.11.021.

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An emerging Panton–Valentine leukocidin-positive CC5-meticillin-resistant *Staphylococcus aureus*-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by whole-genome sequencing

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SUMMARY

Background: A novel Panton–Valentine leukocidin (PVL)-positive meticillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC)5-MRSA-IVc ('Sri Lankan' clone) was recently described from Sri Lanka. Similar isolates caused a recent Irish hospital outbreak.
Aim: To investigate the international dissemination and diversity of PVL-positive CC5-MRSA-IVc isolates from hospital and community settings using whole-genome sequencing (WGS).
Methods: Core-genome single nucleotide polymorphism (cgSNP) analysis, core-genome multi-locus sequence typing (cgMLST) and microarray-based detection of antimicrobial-resistance and virulence genes were used to investigate PVL-positive CC5-MRSA-IVc ($N =$

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Epidemiology
Phylogenomics
PVL
Sri Lankan clone

214 including 46 'Sri Lankan' clone) from hospital and community settings in 12 countries over 17 years. Comparators included 29 PVL-positive and 23 PVL-negative CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V.

Results: Maximum-likelihood cgSNP analysis grouped 209/214 (97.7%) CC5-MRSA-IVc into Clade I; average of 110 cgSNPs between isolates. Clade III contained the five remaining CC5-MRSA-IVc; average of 92 cgSNPs between isolates. Clade II contained seven PVL-positive CC5-MRSA-IVa comparators, whereas the remaining 45 comparators formed an outlier group. Minimum-spanning cgMLST analysis revealed a comparably low average of 57 allelic differences between all CC5/ST5-MRSA-IVc. All 214 CC5/ST5-MRSA-IVc were identified as 'Sri Lankan' clone, predominantly *spa* type t002 (186/214) with low population diversity and harboured a similar range of virulence genes and variable antimicrobial-resistance genes. All 214 Sri Lankan clone isolates and Clade II comparators harboured a 9616-bp chromosomal PVL-encoding phage remnant, suggesting both arose from a PVL-positive meticillin-susceptible ancestor. Over half of Sri Lankan clone isolates were from infections (142/214), and where detailed metadata were available (168/214), most were community associated (85/168).

Conclusions: Stable chromosomal retention of *pvl* may facilitate Sri-Lankan clone dissemination.

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Introduction

Meticillin-resistant *Staphylococcus aureus* (MRSA) contributes significantly to the prevalence of infectious diseases worldwide. Expression of Panton–Valentine leukocidin (PVL), a bicomponent beta-barrel toxin that causes leukocyte lysis or apoptosis via pore formation [1], has been associated with increased MRSA virulence and transmissibility [2–4]. PVL is encoded by the *lukF-PV* and *lukS-PV* genes (known as *pvl*) harboured by lysogenic converting bacteriophages [5]. Carriage of *pvl* was traditionally associated with community-associated (CA)-MRSA, frequently responsible for skin and soft tissue infections (SSTIs), however CA-MRSA lineages are increasingly associated with hospital infection and outbreaks [6–9]. In some cases, highly virulent CA-MRSA with increased transmissibility and greater clonal diversity have surpassed healthcare-associated (HA)-MRSA as the dominant hospital MRSA lineages [7,10,11]. Over the last decade, PVL-positive MRSA clones causing mainly superficial SSTIs have emerged in Irish healthcare and community settings [12,13]. In 2020, the Irish National MRSA Reference Laboratory (NMRSARL) identified *pvl* in 25% of all non-bloodstream infection MRSA submitted for investigation, up from 20% in 2017 and attributed this to an increase in HA-outbreaks [14,15].

McTavish *et al*. recently described a dominant PVL-positive clonal complex (CC) 5 MRSA lineage harbouring a type IVc staphylococcal cassette chromosome *mec* (SCC*mec*) element in Sri Lanka and also identified it in the UK and Australia, referred to hereafter as the 'Sri-Lankan clone' [16]. This lineage was also recently reported in the United Arab Emirates [17]. The emergence of a PVL-positive CC5-MRSA-IVc lineage in Irish hospitals was reported in 2021 [13]. Multiple suspected PVL-positive CA-MRSA outbreaks were investigated and a PVL-positive CC5-MRSA-IVc lineage was identified as being responsible for a 15-month maternity unit outbreak involving 13 patients. The widespread dissemination of novel MRSA lineages with subsequent replacement of predominant clonal types is not uncommon and has been described previously in Ireland and internationally [18–21].

To date, only localized investigations focused on country-specific genomic characterization of PVL-positive ST5-MRSA-IVc isolates, such as the Sri-Lankan clone, have been reported [13,16,17]. This study sought to further investigate the PVL-positive CC5-MRSA-IVc population from Irish hospital and community settings in comparison with a comprehensive collection of similar isolates from 12 countries spanning 17 years using whole-genome sequencing (WGS). WGS provides unrivalled sensitivity and precision for comparing and monitoring the development and spread of historical, current, and emerging clones, as well as tracing infection outbreaks with extremely high resolution.

Methods

MRSA isolates

MRSA isolates ($N = 266$) recovered between 2003 and 2022 were investigated: (1) 214 PVL-positive CC5/sequence type (ST)5-MRSA-IVc isolates (2005–2022) from 12 countries similar to and including 46 previously described Sri-Lankan clone isolates [16] and (2) 52 comparator CC5/ST5-MRSA–SCC–I/II/IVa/IVc/IVg/V isolates (29 PVL-positive and 23 PVL-negative) recovered between 2003 and 2021. Isolates were cryogenically stored at -80 °C. Detailed isolate information and available metadata are provided in Table I and in Supplementary Table S1.

Irish MRSA

All 47 Irish MRSA isolates investigated were submitted to the NMRSARL between 2013 and 2022. These included 14 previously described PVL-positive ST5/t002-MRSA-IVc maternity unit outbreak isolates recovered between 2018 and 2020 that were similar to the Sri-Lankan clone and had a median of three (average:

Table I
Antimicrobial resistance and virulence-associated gene profiles of 214 Panton–Valentine leukocidin (PVL)-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates investigated

Country	Isolates (*N*)	Year(s) of isolation	*spa*-ST-SCC*mec* (*N*)	Antimicrobial resistance genes (*N*)	PVL (+/−)	IEC type (*N*)	Reference
Algeria	Comparator (1)	2003	t450-ST5-IVa	*aadD*, *erm*(C), *fosB*, *lmrP*, *mprF*, *tet*(M), *vga*(A), *sdrM*	+	B	This study
Australia	Sri-Lankan clone (1)	2015	t002-ST5-IVc	*blaZ*, *fosB*, *lmrP*, *sdrM*	+	G	McTavish *et al*. [16]
Czech Republic	Sri-Lankan clone (6)	2018–2021	t002-ST5-IVc	*blaZ* (4), *fosB* (6), *lmrP* (5), *sdrM* (6), *mprF* (6), *erm*(C) (1)	+	G (4) E (1) Novel type 3 (*sak*, *sep)* (1)	This study
	Comparators (2)	2019–2021	t002-ST5-IVa (1) t002-ST5-II (1)	*aadD* (1), *blaZ* (2), *erm*(A) (1), *fosB* (2), *kdpA/B/C/D/E* (1), *lmrP* (2), *mprF* (2), *sdrM* (2), *xylR* (1)	−	G (1) B (1)	
Denmark	Sri-Lankan clone (66)	2007–2021	t002-ST5-IVc	*blaZ* (61), *fosB* (65), *lmrP* (65), *mprF* (65), *sdrM* (66), *erm*(C) (31), *tet*(K) (3), *mupA* (3), *qacA* (3), *qacC* (1), *cat* (1)	+	G (59) F (7)	This study
	Comparators (10)	2013–2015	t002-ST5-IVa (2) t002-ST5-V (8)	*blaZ* (9), *fosB* (10), *lmrP* (10), *mprF* (10), *sdrM* (10), *aacA-aphD* (7), *erm*(C) (2), *tet*(K) (1)	+ (7) − (3)	G (8) F (1) B (1)	
Germany	Sri-Lankan clone (20)	2011–2019	t002-ST5-IVc (15) t535-ST5-IVc (2) t579-ST5-IVc (1) ND-ST5-IVc (2)	*blaZ* (18), *erm*(C) (11), *fosB* (20), *lmrP* (20), *mprF* (20), *sdrM* (19), *tet*(K) (1), *qacA* (1), *msr*(A) (1)	+	G (16) E (1) Novel type 1 (*sep* only) (1) Novel type 2 (*sak*, *scn*, *sea*, *sep*) (1)	This study
	Comparators (2)	2014–2017	t105-ST5-IVc	*blaZ* (1), *fosB* (1), *lmrP* (1), *mprF* (2), *sdrM* (1)	+	B	
Ireland	Sri-Lankan clone (30)	2013–2022	t002-ST5-IVc	*blaZ* (29), *fosB* (30), *lmrP* (30), *mprF* (30), *sdrM* (30), *erm*(C) (5)	+	G (29) None (1)	This study, McManus *et al*. [13]
	Comparator (17)	2013–2019	t002-ST5-I (1) t002-ST5-IVa (2) t002-ST5-IVc (3) t002-ST5-IVg (2) t311-ST5-V (9)	*blaZ* (13), *erm*(C) (10), *fosB* (17), *lmrP* (17), *mprF* (17), *sdrM* (17), *fusC* (10), *fexA* (1), *aadD* (1), *qacA* (1), *merA* (1)	+ (2) − (15)	G (2) F (2) B (3) E (9) Novel type 1 (*sep* only) (1)	
Kuwait	Sri-Lankan clone (1)	2013	t002-ST5-IVc	*blaZ*, *erm*(C), *fosB*, *lmrP*, *mprF*, *sdrM*	+	G	This study
	Comparator (1)	2013	t002-ST5-IVa	*blaZ*, *erm*(C), *fosB*, *lmrP*, *mprF*, *sdrM*	+	G	
Norway	Sri-Lankan clone (24)	2007–2021	t002-ST5-IVc (23) t1062-ST5-IVc (1)	*blaZ* (23), *fosB* (24), *lmrP* (24), *mprF* (24), *sdrM* (24), *erm*(C) (9), *tet*(K) (2), *vga*(A) (1)	+	G (23) Novel Type 2 *(sak*, *scn*, *sea*, *sep)* (1)	This study

	Comparators (12)	2003–2020	t311-ST5-IVa (4) t311-ST5-IVc (2) t002-ST5-IVa (3) t105-ST5-IVc (1) t3089-ST5-IVa (1) t442-ST5-V (1)	*blaZ* (12), *fosB* (12), *lmrP* (12), *mprF* (12), *sdrM* (12), *erm*(C) (1), *tet*(K) (1), *aacA-aphD* (2), *dfrA* (2), *tet*(M) (2), *aphA3* (3), *mph*(C) (1), *msr*(A) (1), *sat* (3), *qacC* (1)	+ (11) − (1)	G (3) B (5) A (4)	
Saudi Arabia	Sri-Lankan clone (4)	2010–2017	t002-ST5-IVc	*blaZ* (3), *fosB* (4), *lmrP* (4), *mprF* (4), *sdrM* (4), *erm*(C) (2), *aphA3* (1), *sat* (1)	+	G (4)	This study
	Comparator (1)	2010	t311-ST5-IVa	*blaZ, fosB, lmrP, mprF, sdrM*	−	B	
Senegal	Comparators (2)	2007	t311-ST5-IVa	*aacA-aphD* (1), *aadD* (1), *blaZ* (1), *dfrA* (2), *fosB* (2), *lmrP* (2), *mprF* (2), *qacC* (1), *tet*(M) (2), *sdrM* (2)	+	B (2)	This study
Slovakia	Comparator (1)	2020	t002-ST5-IVc	*blaZ, fosB, lmrP, mprF, sdrM*	−	B	This study
Sri Lanka	Sri-Lankan clone (33)	2014	t002-ST5-IVc (21) t010-ST5-IVc (1) t045-ST5-IVc (2) t062-ST5-IVc (4) t1062-ST5-IVc (1) ND-ST5-IVc (4)	*blaZ* (33), *fosB* (33), *lmrP* (33), *mprF* (33), *sdrM* (33), *erm*(C) (14), *tet*(K) (4)	+	G (31) Novel type 1 (*sep* only) (1) Novel Type 2 (*sak, scn, sea, sep)* (1)	McTavish *et al.* [16]
Sweden	Sri-Lankan clone (2)	2005–2009	t002-ST5-IVc	*blaZ* (2), *erm*(C) (1), *fosB* (2), *lmrP* (2), *mprF* (2), *sdrM* (2), *tet*(K) (1)	+	G (2)	This study
United Arab Emirates	Sri-Lankan clone (15)	2017–2019	t002-ST5-IVc (12) t010-ST5-IVc (1) t045-ST5-IVc (1) t306-ST5-IVc (1)	*blaZ* (15), *erm*(C) (9), *fosB* (15), *lmrP* (17), *mprF* (17), *sdrM* (17)	+	G (15)	This study
	Comparator (3)	2018	t105-ST5-IVc (2) t002-ST5-IVa (1)	*blaZ* (1), *erm*(C) (1), *fosB* (3), *lmrP* (3), *mprF* (3), *sdrM* (3)	+	G (1) B (2)	
UK	Sri-Lankan clone (12)	2005–2015	t002-ST5-IVc (5) t062-ST5-IVc (2) ND-ST5-IVc (5)	*blaZ* (12), *fosB* (12), *lmrP* (12), *mprF* (12), *sdrM* (12), *erm*(C) (4), *tet*(K) (2), *sat* (1)	+	G (11) D (1)	McTavish *et al.* [16]

Genotypic information for 266 study isolates (Sri-Lankan clone and comparator isolates) recovered from 15 different countries between 2003 and 2022. Sri-Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia, and the Middle East between 2005 and 2022. 214 PVL+ CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates were investigated. The isolates were subjected to whole-genome sequencing and subsequent analyses and profiling to determine antimicrobial resistance gene patterns and virulence gene profiles. Genotypic information was extracted from whole-genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) genotyping, and *S. aureus* Genotyping Kit 2.0 (Abbott) microarray technology [19,23–25]. The isolates also underwent core-genome multi-locus sequence typing and single nucleotide polymorphic analyses.

IEC, immune evasion cluster; ND, not determined – isolates not available and *spa* types could not be determined using *in-silico* techniques on the available genomic sequence data; *sak*, staphylokinase gene; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; *scn*, staphylococcal complement inhibitor; *sea*, staphylococcal enterotoxin a gene; *sep*, staphylococcal enterotoxin p gene; ST, sequence type.

six; range: 0–27) core-genome multi-locus sequence type (cgMLST) allelic differences between isolates [13]. The emergence of this clonal type in Irish hospitals appeared to be recent but a search of the NMRSARL collection for PVL-positive t002-MRSA-IVc and related *spa* types revealed 16 additional isolates recovered between 2013 and 2022 (Table I and Supplementary Table S1). These included two more recent (2021) patient isolates from the maternity unit and 14 patient isolates from nine other hospitals. Seventeen comparator NMRSARL isolates (two PVL-positive ST5-MRSA-IVa and 15 PVL-negative ST5-MRSA-I/IVc/IVg/V) recovered between 2013 and 2019 were also investigated (Table I and Supplementary Table S1).

International MRSA

Additional PVL-positive ST5-MRSA-IVc isolates or WGS datasets from disparate geographical locations were sought for comparison. Contact with international collaborators, an extensive search of the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA)/GenBank, the European Nucleotide Archive (ENA) databases and a literature search (Supplementary Table S2) yielded 219 international isolates or WGS datasets (80 clinical isolates and 139 WGS sequences; Table I and Supplementary Table S1). Of these, 184 were PVL-positive ST5-MRSA-IVc isolates similar to the Sri-Lankan clone, whereas 35 were PVL-positive ($N = 27$) and PVL-negative ($N = 8$) ST5-MRSA-II/IVa/IVc/V comparator isolates.

Clinical isolates

Fifty-six PVL-positive ST5-MRSA-IVc isolates recovered between 2005–2021 in the Czech Republic ($N = 6$), Germany ($N = 4$), Kuwait ($N = 1$), Norway ($N = 24$), Saudi Arabia ($N = 4$), Sweden ($N = 2$) and the United Arab Emirates (UAE) ($N = 15$) underwent WGS at the Dublin Dental University Hospital Microbiology Research Unit (Ireland) (Table I and Supplementary Table S1). Twenty-four ST5-MRSA-II/IVa/IVc/V comparator isolates (19 PVL-positive and five PVL-negative) recovered in Algeria ($N = 1$), the Czech Republic ($N = 2$), Germany ($N = 1$), Kuwait ($N = 1$), Norway ($N = 12$), Saudi Arabia ($N = 1$), Senegal ($N = 2$), Slovakia ($N = 1$) and the UAE ($N = 3$) between 2003 and 2021 were also sequenced (Table I and Supplementary Table S1).

Whole-genome sequences

WGS datasets for 46 previously described PVL-positive ST5-MRSA-IVc Sri-Lankan clone isolates were downloaded from ENA (accession number PRJEB27049) [16]. These patient isolates were recovered in a Sri-Lankan hospital over four months in 2014 ($N = 33$), the UK between 2005 and 2015 ($N = 12$) and Australia in 2015 ($N = 1$) (Table I). WGS datasets for PVL-positive ST5-MRSA-IVc isolates from Denmark (2007–2021) ($N = 66$) and Germany (2011–2019) ($N = 16$) were received. Comparator STST5-MRSA-IVa/IVc/V WGS datasets from Denmark (seven PVL-positive and three PVL-negative; 2013–2015) and Germany (one PVL-positive; 2017) were also included (Table I and Supplementary Table S1).

Genomic DNA extraction and whole-genome sequencing

For short-read sequencing, genomic DNA was extracted and sequencing libraries prepared using the Illumina® DNA Prep Kit (Illumina, Eindhoven, The Netherlands) as described previously [19]. Libraries were scaled to exhibit $\geq$50 $\times$ coverage and sequenced using a 600-cycle MiSeq paired-end Reagent Kit v3 (Illumina) on an Illumina MiSeq sequencer according to the manufacturer's instructions. Short- and long-read datasets for isolates sequenced in Dublin were submitted to the NCBI SRA database under BioProject Nos. PRJNA896922 and PRJNA638834). Short-read datasets for Danish isolates were submitted to the NCBI SRA database under BioProject Nos. PRJNA839593, PRJNA865897, PRJNA869909 and PRJNA898141.

For long-read sequencing, genomic DNA extractions and library preparations were performed as described previously [22]. Sequencing was performed on the MinION platform using a R9.4.1 Flow Cell with the MinKNOW software v20.10 (Oxford Nanopore Technologies, UK) as per manufacturer's instructions.

Hybrid assemblies were performed by genome scaffolding using paired-end short-read and long-read sequences using the Unicycler v0.5.0 pipeline (https://github.com/rrwick/Unicycler). Assembled genomes were annotated using the web-based RAST v2.0 server (https://rast.nmpdr.org) and visualized using Bandage v0.8.1 (https://rrwick.github.io/Bandage/) and SnapGene v6.0.6 (GSL Biotech LLC; https://www.snapgene.com).

Whole-genome sequence analysis

Short-read FASTQ files were assembled, quality assessed and analysed using BioNumerics software (BioNumerics v8.0; Applied Maths, Sint-Martens-Latem, Belgium), Ridom SeqSphere+ software v7.0.4 (Ridom GmbH, Münster, Germany) and web-based SCC*mec*Finder tool (https://cge.cbs.dtu.dk/services/SCCmecFinder/) as described previously [13].

Molecular characterization

DNA microarray profiling was undertaken using the *S. aureus* Genotyping Kit 2.0 (Abbott (Alere Technologies GmbH), Jena, Germany) or WGS analysis. The DNA microarray chip harbours 333 target sequences for approximately 170 antimicrobial-resistance and virulence-associated genes and other genes and sequences that can assign *S. aureus* to CCs and/or STs as described previously [23,24]. WGS-based DNA microarray profiling was undertaken using *in silico* probes of the *S. aureus* Genotyping Kit 2.0. Probe sequences map on to assembled genomes to predict DNA array hybridization patterns [25] and these patterns were compared with *in vitro* array results. Additional investigations into alleles of interest were performed using the Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/), and NCBI BLAST search engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *S. aureus* immune evasion cluster (IEC) types were assigned using microarray profiling. β-haemolysin converting bacteriophages encode combinations of the IEC genes (*sea/sak/scn/sep/chp*), permitting isolates to be clustered into one of eight IEC types based on the combination of genes carried [26].

Phylogenetic analysis

Relatedness between MRSA recovered over an extended time period (2003–2022) was investigated using cgMLST. A

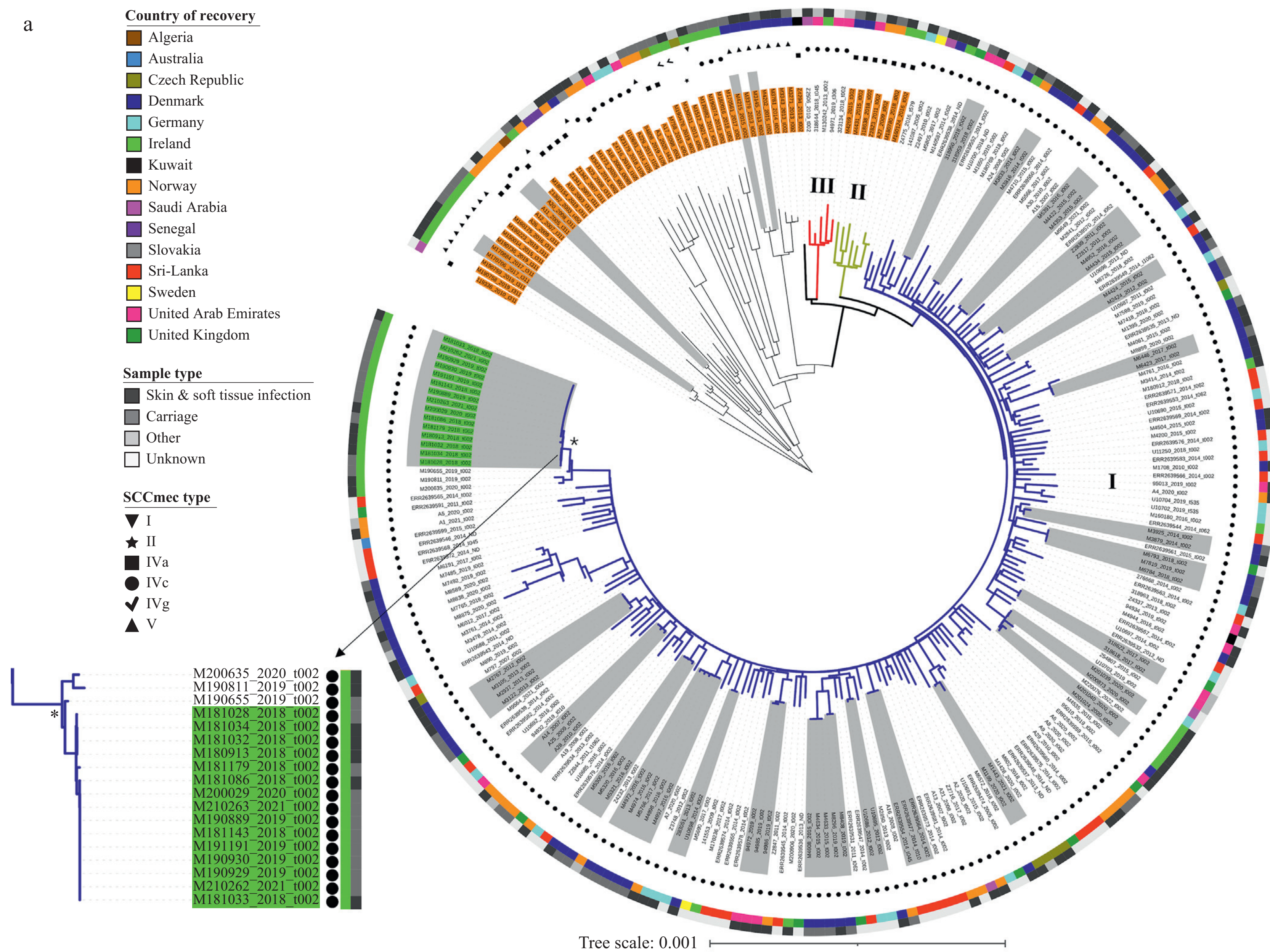

Figure 1. (a) Maximum likelihood tree (MLT) based on phylogenetic analysis of 12,245 core-genome single nucleotide polymorphisms (cgSNPs) for 214 PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional Panton–Valentine leukocidin (PVL)-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Sri-Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia and the Middle East between 2005–2022. Separate node colours/shapes represent country of recovery, sample types and SCC*mec* types as indicated in the legend. Blue branches represent PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates ($N = 209/214$) forming Clade I. Green branches represent PVL-positive CC5/ST5-MRSA-IVa comparator isolates ($N = 7/52$) forming Clade II. Red branches represent the remaining PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates ($N = 5/214$) forming Clade III. The thick black branch represents a PVL-positive CC5/ST5-MRSA-IVa comparator isolate ($n = 1$) branching out next to Clade III. The thin black branches represent the comparator outgroup isolates ($N = 44$) which separate away from Clades I–III. Labels for the 52 comparator isolates are highlighted in orange. Isolate names, year of recovery and *spa* types are all indicated in the branch labelling. Country-specific isolate pairs or clusters containing closely related isolates that differed by ≤10 cgSNPs are shaded in grey. The divergent subgroup of 15 Irish isolates (lacking the *bbp* gene) within the large Sri-Lankan clade (Clade I) is indicated by an asterisk and the isolate names are highlighted in green. The epidemiological and genotypic information for each isolate investigated is provided in Table I and Supplementary Table S1. Corresponding SNP distance matrix data is provided in Supplementary Table S3. (b) Minimum spanning tree (MST) based on core genome multi-locus sequence type (cgMLST) analysis of 1861 target genes for 214 PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Separate node colours represent country of isolation as indicated in the legend. Partitions within nodes represent the presence of ≥2 isolates per node. Comparator isolates are indicated by red squares. Closely related clusters of isolates (≤20 allelic differences to the closest neighbouring isolate within the RIG) are outlined within grey shadowing. Branch numbers indicate the number of allelic differences between neighbouring isolates. Node numbers indicate the 36 related isolate groups (RIGs) in the population (Supplementary Table S1). The subgroup of closely related Irish isolates consisting of 15 isolates with a distinct genotypic profile to other Sri-Lankan clone isolates is outlined in green. The corresponding cgMLST pairwise isolate distance matrix is provided in Supplementary Table S4. The cgMLST-based MST was constructed using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany).

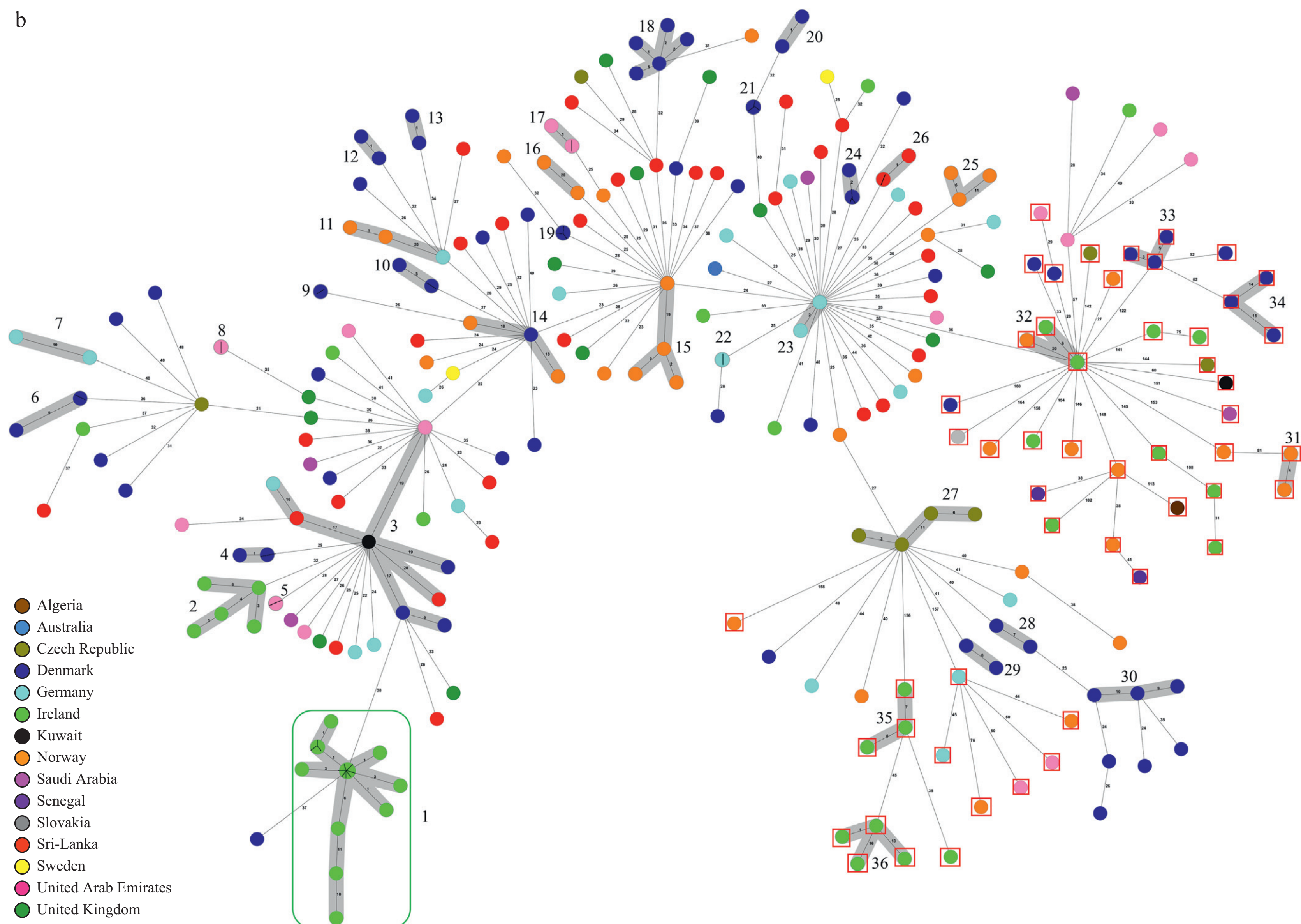

Figure 1. (continued)

minimum spanning tree (MST) based on 1861 core-gene loci was generated for all isolates using the SeqSphere+ (Ridom) cgMLST scheme as previously described [27,28]. Core-genome alignment and variant calling based on single-nucleotide polymorphisms (SNPs) was performed on all isolates mapped against a study-specific reference genome from MRSA isolate 141087 (2005; earliest year of recovery for Sri-Lankan clone isolates investigated), using Snippy v4.6.0 (https://github.com/tseemann/snippy). Recombinant SNPs were removed using Gubbins v3.2.1 (https://github.com/nickjcroucher/gubbins) and a pairwise cgSNP distance matrix generated using snp-dists v3 (https://github.com/tseemann/snp-dists). A cgSNP-based maximum-likelihood tree (MLT) was constructed through IQ-TREE v2.2.0 (http://www.iqtree.org) using recommended IQ-TREE guidelines. The phylogenetic tree was visualized and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

Results

MRSA

MRSA isolates ($N = 266$) recovered between 2003 and 2022 were investigated. These included 214 PVL-positive CC5/ST5-MRSA-IVc isolates from 12 countries similar to and including 46 'Sri-Lankan clone' isolates [16] and 29 PVL-positive and 23 PVL-negative ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates (Table I and Supplementary Table S1). The PVL-positive ST5-MRSA-IVc isolates belonged to eight closely related *spa* types, with t002 predominating (186/214; 86.9%) (Table I and Supplementary Table S1). Six closely related *spa* types were identified among the comparator isolates, half of which were t002 (26/52; 50%) (Table I and Supplementary Table S1).

Phylogenetic analysis of Sri-Lankan clone isolates

To investigate the population structure of Irish PVL-positive CC5/ST5-MRSA-IVc relative to international isolates, all isolates and comparators were subjected to WGS-based phylogenetic analyses. The construction of SNP-based MLT and cgMLST-based MST trees yielded comparable findings regarding isolate relatedness and clustering (Figure 1a, b).

Core-genome SNP analysis

CgSNP analysis based on 12,245 SNPs showed that all 214 PVL-positive CC5/ST5-MRSA-IVc isolates exhibited a pairwise SNP-distance median of 107 (average: 116; range: 0–410) (Supplementary Table S3). The SNP-based MLT grouped the vast majority of isolates (209/214, 97.7%) including all 46 'Sri-

Lankan clone' isolates [16] into one clade, termed Clade I (Figure 1a). Clade I isolates had a median of 106 (average: 110; range: 0–287) SNPs between isolates (Supplementary Table S3). The remaining five PVL-positive ST5-MRSA-IVc grouped into Clade III exhibiting a median of 86 (average: 92; range: 69–127) SNPs (Figure 1a). Clade III differed from Clade I by a median of 232 (average: 237; range: 159–410) SNPs. Most comparator isolates (44/52) formed an outgroup at the base of the MLT (Figure 1a). A single PVL-positive CC5/ST5-MRSA-IVa comparator (isolate Z4294) branched out next to Clade III (thick black branch in Figure 1a). The remaining seven comparators (all PVL-positive ST5/t002-MRSA-IVa) grouped into Clade II, forming the closest neighbour to Clade I (Figure 1a). Clade II differed from Clades I and III by a median of 176 (average: 178; range: 123–331) and 230 (average: 234; range: 207–277) SNPs, respectively (Supplementary Table S3). This tree topology confirmed the identity of all 214 CC5/ST5-MRSA-IVc as 'Sri-Lankan clone'. Hereafter Clades I and III isolates are referred to as 'Sri-Lankan clone'. In general, Sri-Lankan clone isolates did not group according to their country of origin or year of recovery; however, 24 small country-specific clusters of closely related isolates that differed by ≤10 cgSNPs were evident (Figure 1a).

Core-genome MLST analysis

As cgSNP analysis revealed low genotypic diversity among Sri-Lankan clone isolates recovered over 17 years, the previously recommended threshold of ≤24 cgMLST allelic differences for defining closely related isolates [28] was lowered to ≤20. The 214 Sri-Lankan clone isolates exhibited a median of 55 allelic differences from one another (average: 59; range: 0–200) (Supplementary Table S4). The 209 Clade I and five Clade III Sri-Lankan clone isolates exhibited a median of 54 (average: 57; range: 0–153) and 42 (average: 43; range: 28–56) allelic differences, respectively. Clade III isolates differed from Clade I by a median of 116 (average: 117; range: 70–200) allelic differences. Clade II comparator isolates differed from Sri-Lankan clone Clade I and Clade III by a median of 87 (average: 86; range: 36–174) and 106 (average: 93; range: 28–134) allelic differences, respectively (Supplementary Table S4). These findings confirmed limited diversity within the Sri-Lankan clone population.

Thirty-six related isolate groups (RIGs) comprising 123/266 study isolates were evident in the cgMLST-based MST (Figure 1b, Supplementary Table S5). Isolates within each RIG exhibited ≤20 allelic differences to the closest neighbouring isolate in the RIG. Most RIGs included Sri-Lankan clone isolates only (30/36 RIGs) and the remaining six (RIGs 31–36) included comparator isolates only (Figure 1b and Supplementary Table S5). There were 32 country-specific RIGs (27 Sri-Lankan clone isolates only (RIGs 1–2, 4–10, 12–13, 15–30) and five comparator isolates only (RIGs 31, 33–36)) as follows: Denmark ($N = 16$), Norway ($N = 4$), Ireland ($N = 4$), Germany ($N = 3$), UAE ($N = 3$), Sri Lanka ($N = 1$) and Czech Republic ($N = 1$). The remaining four RIGs comprised isolates from two or more countries. RIG-3 comprised eight Sri-Lankan clone isolates from Denmark ($N = 3$), Sri Lanka ($N = 2$), Germany ($N = 1$), Kuwait ($N = 1$) and the UAE ($N = 1$), with an allelic difference range of 6–20 between neighbouring isolates in the RIG and a range of 6–36 allelic differences for the entire RIG (Figure 1b and Supplementary Table S5). Sri-Lankan clone isolates from Norway ($N = 2$) formed two separate RIGs (RIG-11 and RIG-14) with Sri-Lankan clone isolates from Germany ($N = 1$) and Denmark ($N = 1$) with allelic difference ranges of 1–21 and 18–20, respectively. One comparator isolate from Norway formed a third RIG (RIG-32) with two comparator isolates from Ireland.

Irish sub-clade

Potential sub-clades (RIGs 1–36) observed on the cgSNP-based MLT and cgMLST-based MST phylogenetic trees were further investigated using *in silico* DNA microarray profiling and WGS data to identify possible RIG/country-specific characteristics. A genotypic difference was observed between the overall Sri-Lankan clone population and 15/18 Irish Sri-Lankan clone isolates in RIG-1. These 15 isolates formed a distinct Irish sub-clade within the cgSNP-based MLT (Figure 1a) and lacked the *bbp* gene (also known as *sdrE*) encoding a surface-associated, bone sialoprotein-binding protein. The absence of *bbp* in these 15 isolates was confirmed by analysing hybrid assembled genomes (Supplementary Figure S1a).

Genotypic profiling of the Sri-Lankan clone

Strain assignment and antimicrobial resistance genes

DNA microarray profiling, SCC*mec*Finder and Ridom Seqsphere+ template tools for detection of antimicrobial-resistance and virulence-associated genes revealed that most genes in Sri-Lankan clone isolates ($N = 214$) were homogenously distributed (Supplementary Table S1). Microarray analysis grouped Sri-Lankan clone isolates into two categories including 'CC5-MRSA-IVc (*sed/sej/ser*+)' (200/214; 93.5%) and 'CC5-MRSA-IVc (*sed/sej/ser*-)' (14/214; 6.5%). The two groups differed by the presence/absence of the *sed/sej/ser* enterotoxin genes, which were located on a plasmid of approximately 27 kb. Of the five Sri-Lankan clone Clade III isolates (Figure 1a), four isolates were "CC5-MRSA-IVc (*sed/sej/ser*-)" (Supplementary Table S1). The β-lactamase gene *blaZ* and the multi-drug transporter encoding gene *lmrP* mediating resistance to macrolides, lincosamides, streptogramins and tetracycline was harboured by the majority of Sri-Lankan clone isolates (202/214; 94.4% and 212/214; 99.1%, respectively). Antimicrobial genes detected are shown in Table I and Supplementary Table S1.

IEC types

IEC-type G was predominant amongst Sri-Lankan clone isolates (196/214; 91.6%). The remaining isolates harboured IEC-type F (7/214; 3.3%), IEC-type E (2/214; 0.9%), IEC-type D (1/214; 0.5%) or were non-typeable IEC variants harbouring *sep* only (2/214; 0.9%), *sak* and *sep* (1/214; 0.5%) or *sak, scn* and *sea-sep* (4/214; 1.9%). One Sri-Lankan clone isolate (M130242; Supplementary Table S1) lacked lysogenic β-haemolysin converting bacteriophages and carried no IEC genes.

Epidemiological data

Where detailed metadata were available (168/214; 78.5%), just over half of Sri-Lankan clone isolates were CA-MRSA (85/168; 50.6%), while the remainder (50/168; 29.8%) were HA-MRSA or were from hospitalized patients (33/168; 19.6%) (Supplementary Table S1). Most isolates were from infection sites (142/214; 66.3%), with the remainder from carriage (50/214; 23.4%) or unknown sites (22/214; 10.3%). The majority of

infection isolates were from SSTIs (83/142, 58.5%), other infection types (9/142) or were unknown (50/142).

Epidemiological information available for some Sri-Lankan clone isolates from Denmark ($N = 9$), Ireland ($N = 2$) and the UAE ($N = 1$) revealed that these isolates were from patients with international links. The Irish isolates were recovered from patients with a history of travel to Sri Lanka and Turkey, respectively. Within the Danish subset, one patient had been hospitalized in Vietnam, four were from Sri-Lanka and four had travelled to Sri-Lanka. The isolate from the UAE was from a patient from Bangladesh (Supplementary Table S1).

pvl-encoding bacteriophage regions

Clade I and III Sri-Lankan clone ($N = 214$) and Clade II ($N = 7$) comparator isolate short-read assembled genomes were investigated for *pvl*-associated bacteriophage DNA. All isolates harboured the *lukF/S*-PV genes, the phage lysis genes encoding amidase and holin and remnants of phage structural genes encoding the tail fiber and major teichoic acid biosynthesis protein. Genes associated with lysogeny, DNA replication/transcriptional regulation and packaging/structure were not detected [29].

Twenty-six representative Sri-Lankan clone isolates (24 MLT Clade I and two MLT Clade III isolates) available for long- and short-read sequencing underwent hybrid-assembly to further investigate chromosomal regions surrounding *pvl*. These 26/214 isolates (2005–2021) were from Czech Republic ($N = 1$), Denmark ($N = 6$), Germany ($N = 2$), Ireland ($N = 8$), Kuwait ($N = 1$), Norway ($N = 3$), Saudi Arabia ($N = 2$), Sweden ($N = 1$) and the UAE ($N = 2$). Additionally, eight comparator isolates underwent hybrid-assembly (five outgroup and three MLT Clade II comparators, Supplementary Table S1). All 26 Sri-Lankan clone isolates lacked an intact lysogenized *pvl*-encoding phage genome, but harboured a chromosomal remnant encoding the *lukF/S-PV* genes as well as remnants of phage structural and lysis genes (Supplementary Figure S2). In each case, the phage remnant was 9,616 bp, with an intact upstream attachment site (*attL*), but no downstream attachment site (*attR*) (Supplementary Figure S2b). An identical phage remnant was observed in the three Clade II comparator isolates and the single PVL-positive ST5-MRSA-IVa comparator isolate (Z4294) next to Clade III (Figure 1a). The four remaining outgroup comparator isolates all harboured a complete bacteriophage genome of ~45,000 bp which shared 99.99% sequence homology with the well-characterized PVL-encoding phage phiSa2wa (accession no. ON989481.1) (Supplementary Figure S2a) [29]. The phage remnant exhibited 100% sequence homology with the 3′ junction of phage phiSa2wa (Supplementary Figure S2b). Chromosomal sequences adjacent the *pvl*-phage remnant were identical in all isolates investigated by hybrid assembly.

Discussion

The emergence of PVL-positive MRSA is a public health concern globally. These organisms were originally associated with community-onset infections, especially SSTIs but also including necrotizing pneumonia, necrotizing fasciitis, and sepsis [2–4,30,31]. Patients with community onset SSTIs often seek treatment in hospital emergency departments, providing entry routes for CA-MRSA clones into hospitals [32,33]. The spread of PVL-positive CA-MRSA clones into hospitals and resistance to a wide range of antimicrobials is well documented [8,12,34,35].

The increasing prevalence of PVL-positive MRSA isolates from non-bloodstream infections and hospital outbreaks both in Ireland and internationally is concerning [8,13,34,35]. In 2019, McTavish *et al*. characterized a dominant PVL-positive CC5-MRSA-IVc lineage in a Sri Lankan hospital and also identified it in the UK and Australia [16]. In 2021, similar isolates from 13 patients during a protracted Irish maternity unit hospital outbreak were described [13]. Consequently, our investigation sought to compare Irish PVL-positive CC5-MRSA-IVc with the previously reported Sri-Lankan clone and similar international isolates to determine the clone's global distribution, diversity and population structure for the first time.

Phylogenetic analysis of 214 Sri-Lankan clone and 52 comparator isolates revealed that the Sri-Lankan clone is relatively homogenous compared with other PVL-positive CA-MRSA clones that have diverged more significantly over time [35]. Greater diversity maybe revealed in future studies with more disparately recovered isolates. The vast majority of Sri-Lankan clone isolates (209/214, 97.7%) recovered over 17 years grouped into Clade I (Figure 1a) by cgSNP analysis with an average of 110 cgSNPs (57 cgMLST allelic differences) between isolates (Supplementary Tables S3 and S4, respectively). The five remaining Sri-Lankan clone isolates formed Clade III that differed from Clade I by an average of 237 SNPs (117 cgMLST allelic differences). Seven PVL-positive ST5/t002-MRSA-IVa comparator isolates in Clade II formed the closest neighbour to Sri-Lankan Clade I. Segregation of Sri-Lankan clone isolates into country-specific RIGs (27/30 RIGs) by cgMLST probably reflects local transmission and clonal evolution (Supplementary Table S5). Some Danish isolates ($N = 27$) in country-specific RIGs also formed household-specific clusters (RIGs 4, 9–10, 13, 18–21, 24 and 29) (Supplementary Table S1). In some cases, different members of the same household presented with either carriage or infection. Additionally, two isolates recovered from separate patients in a Danish hospital clustered in RIG-30, with 16 cgSNPs (9 cgMLST allelic differences) between isolates (Supplementary Table S1). These findings highlight the significance of CA-MRSA transmission in both community and hospital settings. Only limited inter-country dissemination of closely related Sri-Lankan clone isolates was detected (RIG-3, RIG-11 and RIG-14), although this possibly reflects the limited collection of isolates available for investigation.

Sri-Lankan clone isolates investigated were ST5, predominantly *spa* type t002 or closely related *spa* types and harboured a relatively small number of antimicrobial-resistance genes (Table I and Supplementary Table S1). DNA microarray and WGS data analyses revealed variable IEC gene cluster (*sea/sak/scn/sep/chp*) and plasmid-encoded enterotoxin genes (*sed/sej/ser*) detection, while the majority of other molecular characteristics were highly conserved (Supplementary Table S1). Although IEC-type G (91.6%) was predominant among Sri-Lankan clone isolates, six other IEC types were detected (Supplementary Table S1). Additionally, *sed/sej/ser* enterotoxin genes were absent in only a small number of isolates (6.5%). The absence of the *bbp* gene within Irish maternity unit hospital outbreak-associated isolates (Figure S1a) very likely reflects local loss of the gene as other Irish isolates harboured the gene. Variation in IEC types and enterotoxin

genes probably reflects loss/gain of converting bacteriophages encoding IEC genes and *sed/sej/ser*-encoding plasmids [36,37]. The prevalence of the multi-drug-resistant PVL-negative European CC1-MRSA-IV clone in Ireland [18,19] exemplifies the importance of mobile genetic elements in the successful dissemination of emerging MRSA clones [31,35,38]. Earls *et al.* described the emergence of European CC1-MRSA-IV from a South-Eastern European meticillin-susceptible *S. aureus* (MSSA) CC1 lineage, and its subsequent rapid expansion across Europe in the late 1990s [18,19]. European CC1-MRSA-IV is now the predominant endemic CC1-MRSA clone in Ireland, associated with community transmissions and multi-hospital outbreaks [19]. Periodic replacement of predominant MRSA clones in Irish hospitals is well-documented [39], thus the recovery of the Sri-Lankan clone in 10 Irish hospitals over a nine-year period (2013–2022) is concerning (Table I and Supplementary Table S1).

The Sri-Lankan clone chromosomally integrated defective 9.6 kb *pvl*-encoding phage remnant (Supplementary Figure S2b) may be a useful genetic marker, as the earliest Sri-Lankan clone study isolate (141087, 2005) harboured this remnant. The remnant probably arose by imprecise excision of a lysogenized *pvl*-phage genome, possibly as a result of a fitness cost imposed on the bacteria through carriage of the entire prophage genome [40]. Stable chromosomal *pvl*-retention without phage mobility-associated genes may provide a survival advantage. Defective *pvl*-encoding bacteriophages with truncated tail formation genes have been described in MRSA [41,42]. Furthermore, a defective *pvl*-phage has been reported in the successful CA-MRSA clone USA300 [43]. In the Sri-Lankan clone, approximately 80% of the phage genome has been deleted leaving the *pvl*-encoding remnant.

The seven PVL-positive ST5/t002-MRSA-IVa comparator isolates in Clade II (the closest neighbour to Sri-Lankan Clade I) and the single ST5-MRSA-IVa comparator isolate Z4294 located adjacent to Clade III in the cgSNP MLT also harboured the 9.6 kb phage remnant. These findings suggest that Clade II isolates, isolate Z4294 and Sri-Lankan clone Clade I and III isolates emerged from a PVL-positive common ancestor harbouring the 9.6 kb phage remnant, very likely a PVL-positive ST5-MSSA [19]. Sri-Lankan clone Clade I then went on to disseminate widely. Interestingly, a PVL-positive CC5-MSSA isolate identified in the puBMLST database by *in silico* PCR that also harboured the 9.6 kb phage remnant clustered beside comparator isolate Z4294 and adjacent to Sri Lankan Clade III (Figure 1 and Supplementary Figure S2).

This study had some limitations. Limited Sri-Lankan clone isolates/WGS datasets were recovered following comprehensive literature and WGS database searches making it difficult to assess its true prevalence (Supplementary Table S2). Historical and contemporary data on MSSA progenitor populations is limited in most MRSA lineages [44], including the Sri-Lankan clone. Future investigations require a more comprehensive isolate collection with good quality metadata, including potential progenitor MSSAs from more numerous and disparate regions.

In conclusion, international and local surveillance of emerging MRSA clones is important for monitoring transmission. The association of Sri-Lankan clone isolates with SSTIs in both community and hospital settings in 12 countries spanning 17 years reflects its emergence internationally. The stable chromosomal integration of *pvl* in the Sri-Lankan clone potentially contributes to its dissemination.

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Conflict of interest statement

None of the authors have any conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2022.11.015.

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