Enhanced Typing and Tracking of Methicillin-Resistant *Staphylococcus aureus* and Methicillin-Susceptible *S. aureus* in Irish Hospitals using Whole-Genome Sequencing

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy by

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Declaration

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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a major cause of healthcare-associated and community-associated infection worldwide, with many clones having achieved pandemic status. The development of high-throughput whole-genome sequencing (WGS) has provided the enhanced resolution required to accurately track the spread of MRSA in order to inform more effective infection prevention and control strategies. This technology has also revolutionised investigations of the evolution of established and emerging clones. The work described in this thesis used a variety WGS approaches to comprehensively investigate MRSA isolates from two protracted outbreaks in Irish hospitals and characterised the isolates in relation to international MRSA. The work also used WGS to investigate the transmission dynamics of *S. aureus* among healthcare workers (HCWs) and patients in a large Dublin hospital.

The first part of the study investigated Panton-Valentine leukocidin (PVL)-negative CC1-ST1-MRSA-IV, exhibiting high-level mupirocin-resistance (MupR) mediated by an *ileS2*-encoding conjugative plasmid, recovered from a protracted outbreak in a large Dublin hospital (H1) between 2013 and 2016. The study aimed to (i) characterise the *ileS2*-encoding plasmid, (ii) confirm/dispute a single clonal outbreak and (iii) investigate the relatedness of outbreak isolates to other PVL-negative ST1-MRSA-IV in Ireland. A total of 89 ST1-MRSA-IV isolates from patients (n = 85), HCWs (n = 3) and the environment (n = 1) were investigated. All isolates underwent Illumina MiSeq WGS, DNA microarray profiling and antimicrobial susceptibility testing. The vast majority (78/89) of isolates grouped into one major core-genome multilocus sequence typing (cgMLST)-based minimum spanning tree (MST) cluster, which included two sub-clusters (I and II). Sub-cluster I consisted of 57 isolates with an average of 25 pairwise allelic differences. This included 43/46 H1 isolates, 10/32 isolates from six other healthcare facilities (HCFs) and 4/11 community isolates, all of which were multidrug-resistant (MDR) and 50/57 of which were MupR. The 46 kb *ileS2*-encoding plasmid characterised also harboured *qacA*. Two HCW isolates from sub-cluster I differed from the remaining sub-cluster I isolates by 12-53 pairwise single nucleotide variations (SNVs). Sub-cluster II consisted of 21 isolates with an average of 61 pairwise allelic differences. This included 3/46 H1 isolates, 13/32 isolates from nine other HCFs and 5/11 community isolates, the majority (19/21) of which were MDR. These results identified the outbreak strain in seven HCFs and showed that it constitutes a MupR variant of the predominant ST1-MRSA-IV clone in Ireland. The plasmid-encoded *ileS2* (encoding MupR) and *qacA* (encoding chlorhexidine resistance) genes in the outbreak isolates very likely contributed to maintenance of the outbreak as both mupirocin and chlorhexidine are used routinely for MRSA decolonisation.

The MDR CC1-ST1-MRSA-IV clone identified from the outbreak did not match the characteristics of the only other previously defined PVL-negative ST1-MRSA-IV clone, known as WA MRSA-1. The second part of this study used WGS to investigate the origin of the clone, to determine whether it constitutes a MDR sub-clone of WA MRSA-1 or a distinct, yet uncharacterised, PVL-negative ST1-MRSA-IV clone. Ten CC1-MSSA and 139 CC1-MRSA-IV Irish isolates recovered between 2004 and 2017 (including 89 isolates from the previous part of the study) were investigated. These were compared to 21 German CC1-MRSA, 10 Romanian CC1-MSSA, 10 Romanian CC1-MRSA and two UAE CC1-MRSA, which were selected from an extensive global database, based on similar DNA microarray profiles to the Irish isolates. All isolates underwent Illumina MiSeq WGS and core-genome (cg)SNV analysis. Two PVL-negative clades (A and B1) were identified among four main clades. Clade A included 20 German isolates, 34 Irish isolates, and all Romanian MRSA and MSSA isolates, the latter of which differed from clade A MRSA by 47–130 cgSNVs. Clade B1 included the remaining German isolate, 17 Irish isolates and the two UAE isolates, all of

which corresponded to the WA MRSA-1 clone based on genotypic characteristics. MRSA within clades A and B1 differed by 188 cgSNVs and clade-specific SCC*mec* characteristics were identified, indicating independent acquisition of SCC*mec*. These results identified a European PVL-negative CC1-MRSA-IV clone that is distinctly different from WA MRSA-1, and which may have originated in South-Eastern Europe.

From 2009 to 2011 (transmission period [TP] 1) and 2014 to 2017 (TP2), two outbreaks involving CC88-MRSA *spa* types t186 and t786, respectively, occurred in the neonatal intensive care unit (NICU) of an Irish hospital. The third part of this study used WGS to investigate the relatedness of these isolates, their relatedness to other CC88 MRSA in Ireland, and their likely geographic origin. A total of 28 Irish CC88-MRSA isolates recovered between 2009 and 2017 were investigated, including 20 patient and two HCW isolates from the outbreak hospital, and six patient isolates from four other hospitals. These were compared to 13 international isolates, selected from an extensive global database based on similar DNA microarray profiles to the Irish isolates. The majority (25/28) of Irish isolates (including those from the outbreak hospital and two additional hospitals) were identified as ST78-MRSA-IVa and formed a large cluster, exhibiting 1–71 pairwise allelic differences, in a whole-genome (wg)MLST-based MST. The TP2 isolates were characterised by a different *spa* type and the loss of *hsdS*. The three remaining Irish isolates were identified as ST88-MRSA-IVa and dispersed at the opposite end of the MST, exhibiting 81–211 pairwise allelic differences. Core-genome MLST and sequence-based plasmid analysis revealed the recent shared ancestry of Irish and Australian ST78-MRSA-IVa, and of Irish and French/Egyptian ST88-MRSA-IVa. These results revealed the homogeneity of isolates from the two NICU outbreaks, HCW involvement in the outbreak and the presence of the outbreak strain in two other Irish hospitals.

HCWs have been repeatedly linked to *S. aureus* outbreaks, however, routine HCW screening for MRSA is not performed in Irish hospitals. The aim of the fourth part of this study was to use WGS to investigate whether HCWs are a significant source of *S. aureus* in patients in non-outbreak scenarios. Additionally, as the rate of MSSA among *S. aureus* causing invasive infections in Ireland is increasing, the secondary aim of this study was to investigate the *S. aureus* (MSSA and MRSA) population in a large Dublin hospital, using WGS. Oral and nasal samples from patients and HCWs, and clinical *S. aureus* isolates, were collected on nine wards of an Irish hospital over six months. Near-patient environmental and air sampling was also performed. MRSA and MSSA were detected using SASelect/MRSASelect chromogenic agars and routine identification methods. All isolates underwent Illumina MiSeq WGS. *Staphylococcus aureus* was recovered from 35.6% of HCWs, 22.7% of patients, 15.2% of near-patient areas and 20.7% of air sampling sites. MRSA was recovered from 3.4% of HCWs, 1.5% of patients, 0% of near-patient areas and 6.0% of air sampling sites. The ST45 (16.1%), ST30 (14.9%) and ST5 (11.5%) lineages were particulary prevalent among both patients and HCWs. Based on wgMLST, 15 transmission cases were identified involving ~35% of all isolates investigated. A total of 3/30 *S. aureus*-positive patients harboured a strain closely related to that of a HCW, and 3/53 *S. aureus*-positive HCWs harboured a strain closely related to that of a patient. These results indicated that HCWs rarely transmit *S. aureus* to patients in non-outbreak settings, suggesting that routine screening of HCWs for MRSA may not be benefical in MRSA-endemic settings.

This study used WGS to detect and characterise a MDR MRSA clone emerging in Europe, investigate two protracted MRSA outbreaks, identify a novel *ileS2* and *qacA*-encoding plasmid and elucidate the transmission dynamics of *S. aureus* in a large Dublin hospital. Harmonised efforts must be made to facilitate the widespread establishment of WGS technologies in surveillance and clinical microbiology laboratories so that they may be used to directly benefit public health and patient care in real-time.

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Abbreviations

BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
bp	Base pair
BSI	Blood stream infection
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CBA	Columbia blood agar
CC	Clonal complex
cgMLST	Core-genome multilocus sequence typing
cgSNV	Core-genome single nucleotide variation
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive/variable staphylococci
CRE	Carbapenem-resistant *Enterobacteriaceae*
CWA	Cell wall-anchored
ddNTP	Dideoxynucleotide triphosphates
DDUH	Dublin Dental University Hospital
DLV	Double locus variant
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EARS-Net	European Antimicrobial Resistance Surveillance Network
eBURST	Based Upon Related Sequence Types
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia*; for example
et al.	*Et alia*; and others
EU	European Union
EUCAST	European Committee of Antimicrobial Susceptibility Testing
g	Gravitational force
Gb	Gigabase

GP	General medical practitioner
h	Hour
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HCF	Healthcare facility
HCW	Healthcare worker
HRP	Horseradish-peroxidase
ICU	Intensive care unit
IEC	Immune evasion complex
IPC	Infection prevention and control
IS	Insertion sequence
IUPAC	International Union of Pure and Applied Chemistry
J	Joining
kb	Kilobase
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
M	Molar
Mb	Megabase
MDR	Multidrug-resistant
MFS	Major Facilitator Superfamily
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
MLS_B	Macrolides, lincosamides and streptogramin B
MLST	Multilocus sequence typing
MLTs	Maximum likelihood tree
MPTs	Maximum parsimony tree
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecules

MSSA	Methicillin-susceptible *Staphylococcus aureus*
MST	Minimum spanning tree
MupR	High-level mupirocin-resistant
NCBI	National Centre for Biotechnology Information
NGS	Next generation sequencing
NICU	Neonatal intensive care unit
NJT	Neighbour joining tree
NMRSARL	National methicillin-resistant *Staphylococcus aureus* reference laboratory
ONT	Oxford nanopore technology
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Potential hydrogen
PSM	Phenol-soluble modulins
PVL	Panton-Valentine leukocidin
QAC	Quaternary ammonium compounds
qRT-PCR	Quantitative real-time polymerase chain reaction
s	Seconds
SAG	Super antigens
SaPI	*Staphylococcus aureus* pathogenicity islands
SCC	Staphylococcal cassette chromosome
SLV	Single locus variant
SMR	Small Multidrug Resistance
SMRT	Single-molecule real-time
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SPRI	Solid Phase Reversible Immobilization

SSTI	Skin and soft tissue infection
ST	Sequence type
TBE	Tris-borate/EDTA
TP	Transmission period
TSA	Trypticase Soy Agar
UAE	United Arab Emirates
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic
USA	United States of America
VNTR	Variable-number tandem-repeat
w/v	Weight per volume
WA	Western Australia
wgMLST	Whole-genome multilocus sequence typing
WGS	Whole-genome sequencing
ZMW	Zero-mode waveguides
ºC	Degrees celsius
µg	Microgram
µg	Microlitre
%	Percentage
≥	Greater than or equal to
≤	Less than or equal to
>	Greater than
<	Less than
n	Number
α	Alpha
β	Beta
δ	Delta
′	Prime

Publications

Some of the work presented in this thesis has been published in refereed international publications, as listed below. Offprints of the manuscripts are included at the end of the thesis (Appendix 1).

Earls, M. R., Kinnevey, P. M., Brennan, G. I., Lazaris, A., Skally, M., O'Connell, B., et al. (2017). The recent emergence in hospitals of multidrug-resistant community-associated sequence type 1 and *spa* type t127 methicillin-resistant *Staphylococcus aureus* investigated by whole-genome sequencing: Implications for screening. *PLoS One* 12, 1–17. doi:10.1371/journal.pone.0175542.

Earls, M. R., Coleman, D. C., Brennan, G. I., Fleming, T., Monecke, S., Slickers, P., et al. (2018). Intra-hospital, inter-hospital and intercontinental spread of ST78 MRSA from two neonatal intensive care unit outbreaks established using whole-genome sequencing. *Front. Microbiol.* 9, 1485. doi:10.3389/fmicb.2018.01485.

Earls, M. R., Shore, A. C., Brennan, G. I., Simbeck, A., Schneider-Brachert, W., Vremeră, T., et al. (2019). A novel multidrug-resistant PVL-negative CC1-MRSA-IV clone emerging in Ireland and Germany likely originated in South-Eastern Europe. *Infect. Genet. Evol.* 69, 117–126. doi:10.1016/j.meegid.2019.01.021.

Chapter 1

General Introduction

1.1 *Staphylococcus aureus*

Staphylococcus is a genus of Gram-positive, facultatively anaerobic bacteria, which commensally inhabits the skin and mucosal membranes of humans and animals. Staphylococcal species can be divided into two main groups based on their ability to express coagulase, an extracellular protein that enables the clotting of fibrin in blood plasma (Foster, 1996). Coagulase-negative staphylococci (CoNS) are a leading cause of hospital-acquired infections, particularly those associated with indwelling or implanted medical devices (Becker et al., 2014). Coagulase-positive/variable staphylococci (CoPS) are a major cause of both hospital- and community-acquired infections (Foster, 1996). *Staphylococcus aureus* is the most clinically relevant CoPS species (Foster, 1996). Upon breaching the host defences, *S. aureus* is capable of causing a wide variety of infections which range in severity from superficial skin and soft tissue infections (SSTIs), to life-threatening invasive infections (Foster, 1996).

1.1.1 *Staphylococcus aureus* carriage

Colonisation with *S. aureus* is an important risk factor for subsequent infection (Hortal et al., 2008). The anterior nares are the primary colonisation site of *S. aureus*. The skin, perineum and pharynx also commonly harbour *S. aureus*, while the gastrointestinal tract, vagina and axillae are less frequently colonised (Wertheim et al., 2005). Cross-sectional studies suggest that *S. aureus* nasal carriage rates vary among geographic locations (Sollid et al., 2014). In the USA and UK, approximately 30% of the population harbour nasal *S. aureus* at a given time (Gamblin et al., 2013; Gorwitz et al., 2008). It is likely, however, that this rate varies between sample groups as higher carriage rates have been detected in subpopulations such as children, while lower rates have been observed among other groups, such as smokers (Sollid et al., 2014; Wertheim et al., 2005).

Longitudinal studies have revealed the complexity of *S. aureus* carriage, the dynamics of which are not yet fully understood. Traditionally, individuals have been categorised as persistent carriers (who almost always carry a single strain), intermittent carriers (who sporadically harbour various strains) or non-carriers (who almost never harbour *S. aureus*) (Kluytmans et al., 1997). More recently, however, it has been demonstrated that non- and intermittent carriers can be grouped together based on elimination kinetics, nasal load and anti-staphylococcal antibody titre levels (van Belkum et al., 2009). Furthermore, a recent study identified just two carriage patterns among 1123 adults; highly transient carriage and long-term carriage, the latter of which did not necessarily involve the

maintenance of a single strain (Miller et al., 2014). This study also determined that strain type influenced the length of time for which an individual was colonised (Miller et al., 2014), highlighting the importance of bacterial determinants of carriage, in addition to host determinants.

1.1.2 *Staphylococcus aureus* transmission

Human carriers are the most important source of *S. aureus* among the human population, although animals also constitute a significant reservoir. *Staphylococcus aureus* is typically transmitted between individuals via direct physical contact, or through an environmental intermediary, such as a door handle (Sollid et al., 2014). Acquisition may also occur via droplets in the air, following dispersion from the nose or mouth of a carrier (Gehanno et al., 2009). In hospitals, patients are at risk of acquiring *S. aureus* from other patients, visitors and healthcare workers (HCWs). Studies have indicated that HCWs with high levels of patient contact exhibit increased nasal carriage rates (Sollid et al., 2014; Suffoletto et al., 2008) and in recent years, HCWs have been definitively linked to hospital outbreaks of *S. aureus* (Harris et al., 2013; Roisin et al., 2016).

1.2 The *S. aureus* genome

In silico analysis has revealed that the *S. aureus* genome is approximately 2.8 Mb in size, exhibits a GC content of ~33%, and includes approximately 2,800 protein-encoding sequences (https://www.ncbi.nlm.nih.gov/genome/?term=staphylococcus+aureus). In order to describe evolutionary changes and phylogenetic relationships, the *S. aureus* genome can be divided into the core-genome and the accessory genome.

1.2.1 The core-genome

The core genome accounts for approximately 85% of the entire *S. aureus* genome and includes both stable core genes (~75%) and core-variable genes (~10%) (Mccarthy et al., 2012). The stable core genome is highly conserved among all *S. aureus* strains and consists of genes associated with housekeeping functions such as central metabolism, growth and survival. Conversely, the core-variable genome comprises a subset of lineage-specific genes exhibiting reduced stability. These genes encode non-essential growth/survival determinants and species-specific virulence factors (Howden et al., 2008). Genetic diversity in the core-genome derives from single nucleotide variations (SNVs) and insertions/deletions (indels). When a SNV becomes fixed within a population, it is referred to as a single nucleotide polymorphism (SNP). SNPs which do not change the encoded

amino acid (synonymous SNPs) are phenotypically silent, while non-synonymous SNPs may result in functional effects. The latter includes mutations which may be retained under the selective pressure exerted on clinical strains by the use of antibiotics (Lindsay and Holden, 2006). For example, point mutations in *gyrA* and *fusA* confer ciprofloxacin and fusidic acid resistance, respectively (Besier et al., 2003; Sreedharan et al., 1990). Indels can also have significant functional effects on a strain. They range in size from one nucleotide to several kilobases and may include complete genes or operons (Lindsay and Holden, 2006).

1.2.2 The accessory genome

The accessory genome constitutes the remaining ~15% of the *S. aureus* genome, and contains genes associated with non-essential functions such as virulence and resistance (Mccarthy et al., 2012). These genes are typically located on mobile genetic elements (MGEs) which can be transferred horizontally between cells via conjugation, transduction or transformation (Fig. 1.1) (Lindsay, 2014). Five distinct types of MGE have been described in *S. aureus*, namely, lysogenic bacteriophage, plasmids, *S. aureus* pathogenicity islands (SaPIs), staphylococcal cassette chromosome (SCC) elements and transposons. Lysogenic bacteriophages and SaPIs generally harbour virulence genes, while resistance genes typically reside on plasmids, transposons and SCC elements (Lindsay and Holden, 2006).

1.2.2.1 Plasmids

Plasmids are self-replicating DNA molecules that can integrate into the chromosome or exist as free circularised DNA (Malachowa and Deleo, 2010). The plasmids of *S. aureus* are typically divided into three different classes: (i) small (<5 kb) multicopy plasmids which are cryptic or carry a single resistance gene, (ii) larger (usually 15-30 kb) low copy number plasmids that can carry more than one resistance gene, and (iii) large (30-60 kb) plasmids that can carry several resistance genes and that harbour the *tra* locus, which mediates conjugative transfer (Baba-moussa, 2014). The resistance genes located on these large conjugative plasmids are often encoded by transposons that are flanked by insertion sequences (IS) that mediate their mobility (Baba-moussa, 2014). For example, IS*257* typically flanks the *ileS2* gene (which encodes high-level mupirocin resistance), forming a transposon that is generally located on a large conjugative plasmid (Pérez-Roth et al., 2010).

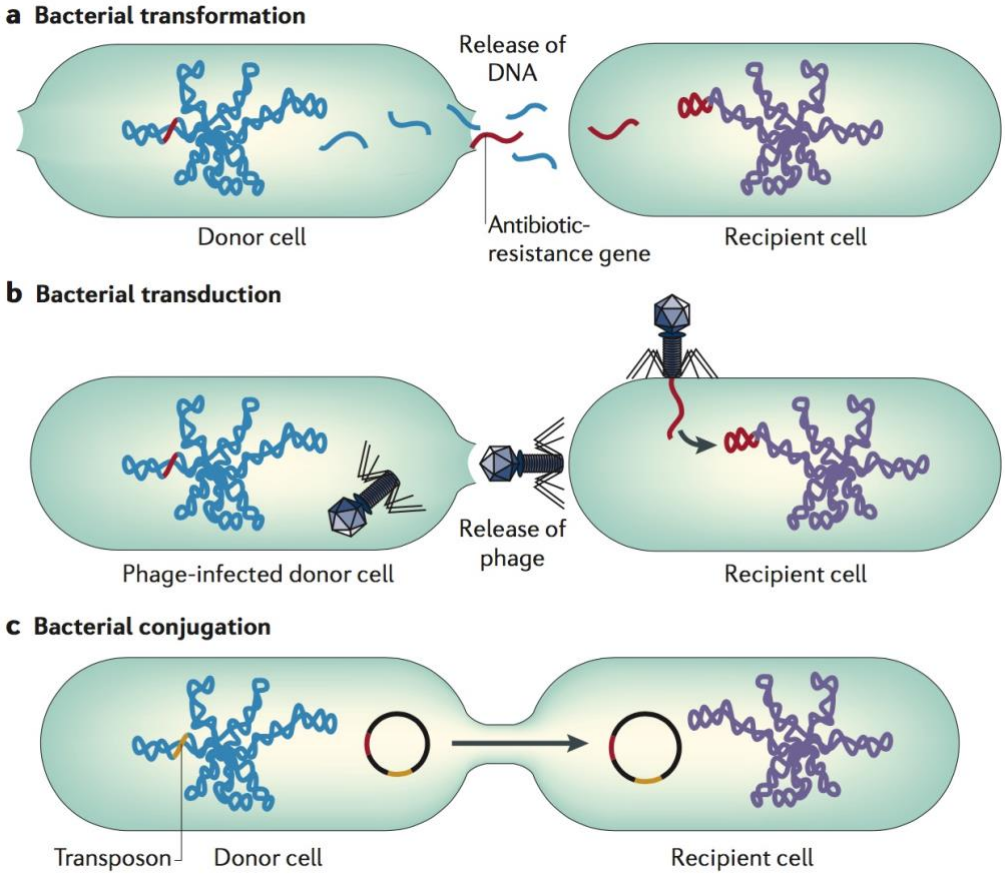


Figure 1.1 Horizontal gene transfer between bacteria. **(a)** Transformation occurs when DNA is released following cell lysis and subsequently acquired by another cell, which has been rendered competent to take up exogenous DNA. The newly acquired DNA can be integrated into the chromosome or plasmid of the recipient cell by recombination and/or transposition. **(b)** During transduction, fragments of bacterial DNA are transferred between cells via bacteriophage. Some bacteriophage harbour the integrase enzyme required to mediate the site- and orientation-specific integration of the linear phage genome into the bacterial chromosome (lysogeny). **(c)** During conjugation, bacterial cells make direct contact and a mating bridge is formed through which DNA is exchanged. Transposons are sequences of DNA which harbour insertion sequences that mediate their mobility, and which are often found on large plasmids. Adapted from Furuya and Lowy, 2006.

1.3 Adhesion, immune evasion and virulence in *S. aureus*

Staphylococcus aureus harbours a diverse range of virulence-associated genes which are distributed between the core and accessory components of the genome (Table 1.1). These genes encode proteins that enhance the pathogenic potential of *S. aureus* at different stages of the infection process. The core genome encodes an array of cell wall-anchored (CWA) proteins, a selection of which termed microbial surface components recognising adhesive matrix molecules (MSCRAMMs), mediate adherence to host proteins (Foster et al., 2014). These include Cna which binds collagen, FnBPA/B which bind fibronectin, and ClfA/B which bind fibrinogen (Burke et al., 2011; Deivanayagam et al., 2002; Ganesh et al., 2011; Keane et al., 2007; Zong et al., 2005). Other CWA proteins promote biofilm formation (e.g. Bap) or facilitate immune evasion (e.g. protein A) (Cedergren et al., 1993; Li et al., 2012).

In addition to CWA proteins, *S. aureus* can express a broad range of cytolytic toxins, the majority of which are encoded by the accessory genome (Mccormick et al., 2001). These include both receptor-mediated toxins (e.g. α-haemolysin, the Panton Valentine leukocidin [PVL] and other leukocidins) and non-specific cytolytic toxins known as phenol-soluble modulins (PSMs; e.g. δ-haemolysin) (Otto, 2014). *Staphylococcus aureus* can also express a variety of superantigens (SAGs). Bacterial SAGs are small exoproteins that disrupt host immune system by binding class II major histocompatibility complex molecules and T-cell receptors. This stimulates the uncontrolled activation of T lymphocytes which can lead to potentially fatal toxic shock syndrome (Mccormick et al., 2001). The SAG family comprises at least 26 genetically distinct members, including the toxic shock syndrome toxin, staphylococcal enterotoxins (SEs) and SE-like toxins (Tuffs et al., 2018). *Staphylococcus aureus* can also produce exfoliative toxins A, B and D, which are serine proteases that damage the superficial epidermis (Grumann et al., 2014).

The *S. aureus* accessory-genome can also harbour genes encoding the immune-modulating proteins, chemotaxis inhibitory protein of staphylococci (CHIPS), staphylococcal complement inhibitor (SCIN) and staphylokinase (Sak) (Malachowa and Deleo, 2010). Both SCIN and Sak function primarily to prevent opsonisation (the labelling of cells for

Table 1.1 Examples of virulence factors expressed by *S. aureus*

Virulence Factor	Gene(s)	Biological effect
Adhesion		
Collagen binding protein	*cna*	Binds collagen in host tissue
Fibrinogen binding protein	*clfA, clfB*	Binds fibrinogen in host tissue
Fibronectin binding protein	*fnbpA, fnbpB*	Binds fibronectin in host tissue
Damage to host cells and tissues		
α-haemolysin	*hla*	Cytolytic pore-forming toxin
β-haemolysin	*hlb*	Hydrolyses sphingomyelin
Enterotoxins	*sea-seu*	Uncontrolled activation of T lymphocytes
Exfoliative toxins	*etA, etB, etD*	Disrupt epidermal layers
Leukocidins	*lukD/E, lukA/B*	Pore-forming leukocyte toxin
Lipase	*lip*	Hydrolyses lipids
Panton-Valentine leukocidin	*lukF/S-PV*	Pore-forming leukocyte toxin
Toxic shock syndrome toxin	*tsst-1*	Uncontrolled activation of T lymphocytes
Immune evasion		
Capsular polysaccharide	*cap*	Inhibits chemotaxis and phagocytic engulfment
Immune evasion complex	*chp, sak, scn, sea, sep*	Inhibits immune response
Protein A	*spa*	Inhibits opsonisation
Persistence		
Arginine catabolic mobile element	*arcA, arcB, arcC, arcD*	Facilitates persistent colonisation
Biofilm-associated protein	*bap*	Facilitates biofilm formation
S. aureus surface protein X	*sasX*	Facilitates biofilm formation

phagocytosis), while CHIPS prevents the migration of neutrophils and monocytes towards the site of infection, thus indirectly preventing phagocytosis (de Haas et al., 2004; Jin et al., 2004; Rooijakkers et al., 2005). The genes encoding CHIPS, SCIN and Sak (*chp*, *scn* and *sak*, respectively), together with those encoding SEA and SEP (*sea* and *sep*, respectively), are carried by a single family of lysogenic converting bacteriophages (*hlb*-converting phages) that insert into, and inactivate, the β-haemolysin gene, *hlb*. Any combination of these five genes is referred to as the human immune evasion complex (IEC), of which eight variants (A-H) have been defined, to date (Table 1.2) (Carroll et al., 1995; Coleman et al., 1989; Dempsey et al., 2005; Wamel et al., 2006). The presence of the IEC can indicate that a lineage has co-evolved primarily with humans, as *hlb* confers a selective advantage in non-human hosts (Mrochen et al., 2017; Schmidt et al., 2017).

1.4 The emergence of antibiotic resistance in *S. aureus*

Antibiotic resistance was first detected in *S. aureus* in the early 1940s, shortly after the introduction of penicillin into clinical practice (Barber and Rozwadowska-Dowzenko, 1948). Initially confined to hospitals, penicillin-resistant strains later emerged in the community, and were pandemic by the 1950s (Rountree and Freeman, 1955). Penicillin-resistant *S. aureus* express a penicillinase, which hydrolyses the β-lactam ring of penicillin, thus eliminating its antimicrobial activity (Richmond, 1975). To circumvent this resistance mechanism, a semisynthetic penicillin derivative termed methicillin, was introduced into clinical practice in 1959. Just two years later, however, methicillin-resistant *S. aureus* (MRSA) were identified in the UK (Jevons, 1961), and by the 1980s, MRSA were pandemic in hospitals worldwide (Chambers and Deleo, 2009). This resulted in the increased use of other antibiotics to treat *S. aureus* infections and MRSA exhibiting resistance to additional antibiotic classes subsequently became prevalent. Table 1.3 provides a list of antimicrobial agents to which *S. aureus* often exhibits resistance.

1.4.1 Methicillin resistance in *S. aureus*

Methicillin is a penicillinase-resistant β-lactam that prevents cell wall synthesis by inhibiting the activity of the four native staphylococcal penicillin-binding proteins (PBPA-D). In *S. aureus*, PBPs catalyse the synthesis and crosslinking of peptidoglycan in the bacterial cell wall via transglycosylation and transpeptidation, respectively (Sauvage et al., 2008). In methicillin-susceptible *S. aureus* (MSSA), methicillin binds PBP and inhibits its activity. In MRSA, however, alternate PBPs (PBP2a or PBP2′) with reduced β-lactam

Table 1.2 The immune evasion cluster types identified in *S. aureus*[a]

Immune evasion cluster type	Gene(s) present[b]
A	*chp, sak, scn, sea*
B	*chp, sak, scn*
C	*chp, scn*
D	*sak, scn, sea*
E	*sak, scn*
F	*chp, sak, scn, sep*
G	*sak, scn, sep*
H	*scn*

[a]Adapted from Wamel et al., 2006.

[b]*chp*, chemotaxis inhibitory protein of staphylococci; *sak*, staphylokinase; *scn*, staphylococcal complement inhibitor; *sea*, staphylococcal enterotoxin A; *sep*, staphylococcal enterotoxin P

Table 1.3 Examples of acquired antimicrobial resistance genes identified in *S. aureus*

Antimicrobial agent/class	Target	Resistance mechanism	Associated gene(s)
Aminoglycosides	30S ribosomal subunit	Enzymatic inactivation of antimicrobial agent	*aacA-aphD, aadD, aphA3*
Antiseptics	Cell membrane	Active efflux	*qacA, qacB, qacC*
β-lactams	Penicillin-binding proteins	Enzymatic inactivation of antimicrobial agent	*blaZ*
Chloramphenicol	23S ribosomal subunit	Enzymatic inactivation of antimicrobial agent	*cat*
Fusidic acid	Elongation factor G	Blocking of target site	*fusB, fusC*
Glycopeptides	D-ala-D-ala of peptidoglycan	Alternative target protein with low affinity for antimicrobial agent	*vanA, vanB*
Linezolid	23S ribosomal subunit	Target site modification	*cfr*
Macrolides, lincosamides and streptogramin B compounds	50S ribosomal subunit	Target site modification	*erm*(A), *erm*(B), *erm*(C)
		Enzymatic inactivation of antimicrobial agent	*msr*(A), *mph*(C), *lnu*(A)
Methicillin	Penicillin-binding proteins	Alternative target protein with low affinity for antimicrobial agent	*mecA, mecB, mecC*
Mupirocin	Isoleucyl tRNA synthetase	Alternative target protein with low affinity for antimicrobial agent	*ileS2, ileS3*
Spectinomycin	30S ribosomal subunit	Enzymatic inactivation of antimicrobial agent	*spc*
Tetracycline	30S ribosomal subunit	Active efflux	*tet*(K), *tet*(L)
		Target site modification	*tet*(M), *tet*(O)
Trimethoprim	Dihydrofolate reductase	Alternative target protein with low affinity for antimicrobial agent	*drfS1, dfrD, dfrK*

affinity, evade the usual activity of methicillin and continue catalysing cell wall synthesis (Hartman and Tomasz, 1984). These alternate PBPs are typically encoded by *mecA*, and less often *mecC*, both of which carried on SCC elements termed SCC*mec*. Recently, a third *mec* homolog (*mecB*) was identified on a plasmid in a *S. aureus* isolate in Germany (Becker et al., 2018). Interestingly, it has been shown that MRSA harbouring SCC*mec* existed over 14 years before the introduction of methicillin into clinical practice, indicating that the widespread use of penicillin was instrumental in selecting for methicillin resistance, in addition to penicillin resistance (Harkins et al., 2017).

1.4.1.1 SCCmec

SCC*mec* elements range in size from approximately 20-60 kb, and integrate into the chromosome at the 3′ end of *orfX*, which encodes a ribosomal transferase (Shore and Coleman, 2013). Each SCC*mec* element includes a *mec* gene complex, a cassette chromosome recombinase (*ccr*) gene complex, and three joining (J) regions. The *ccr* complex comprises one or two *ccr* genes of four main types (*ccrA*, *ccrB*, *ccrC* and *ccrAA*) and various allotypes, and surrounding open reading frames (ORFs). While the function(s) of these ORFs remains unclear, the *ccr* genes are known to encode serine recombinases that mediate site- and orientation-specific integration into and excision from the chromosome. The *mec* gene complex includes *mecA* or *mecC*, regulatory genes *mecR1* and *mecI*, when present, and associated insertion sequences. Five different classes (A-E) of the *mec* gene complex have been identified in *S. aureus*, to date, four of which include *mecA* (classes A-D) and one of which includes *mecC* (class E). The J regions are the genomic regions surrounding the *ccr* and *mec* gene complexes. These are non-essential components of the cassette which are highly variable and which may harbour additional antimicrobial resistance determinants (Ito et al., 2009). To date, 13 different SCC*mec* types (I-XIII) have been identified in *S. aureus*, each of which harbours a unique *ccr* and *mec* gene complex combination (Fig. 1.2) (Baig et al., 2018; Ito et al., 2009; Wu et al., 2015). Many different subtypes have also been defined, based on variation in the J regions (Monecke et al., 2016b).

Sequence-based studies indicate that both SCC and the *mecA* complex evolved largely in *Staphylococcus sciuri*, a CoNS species prevalent in animals (Rolo et al., 2017b; Tsubakishita et al., 2010). Evidence suggests that *mecA* evolved from PBPD, and subsequently acquired regulatory genes to form the *mec* complex. The *mec* complex was then integrated into SCC, which had evolved independently, at *orfX* (Rolo et al., 2017b).

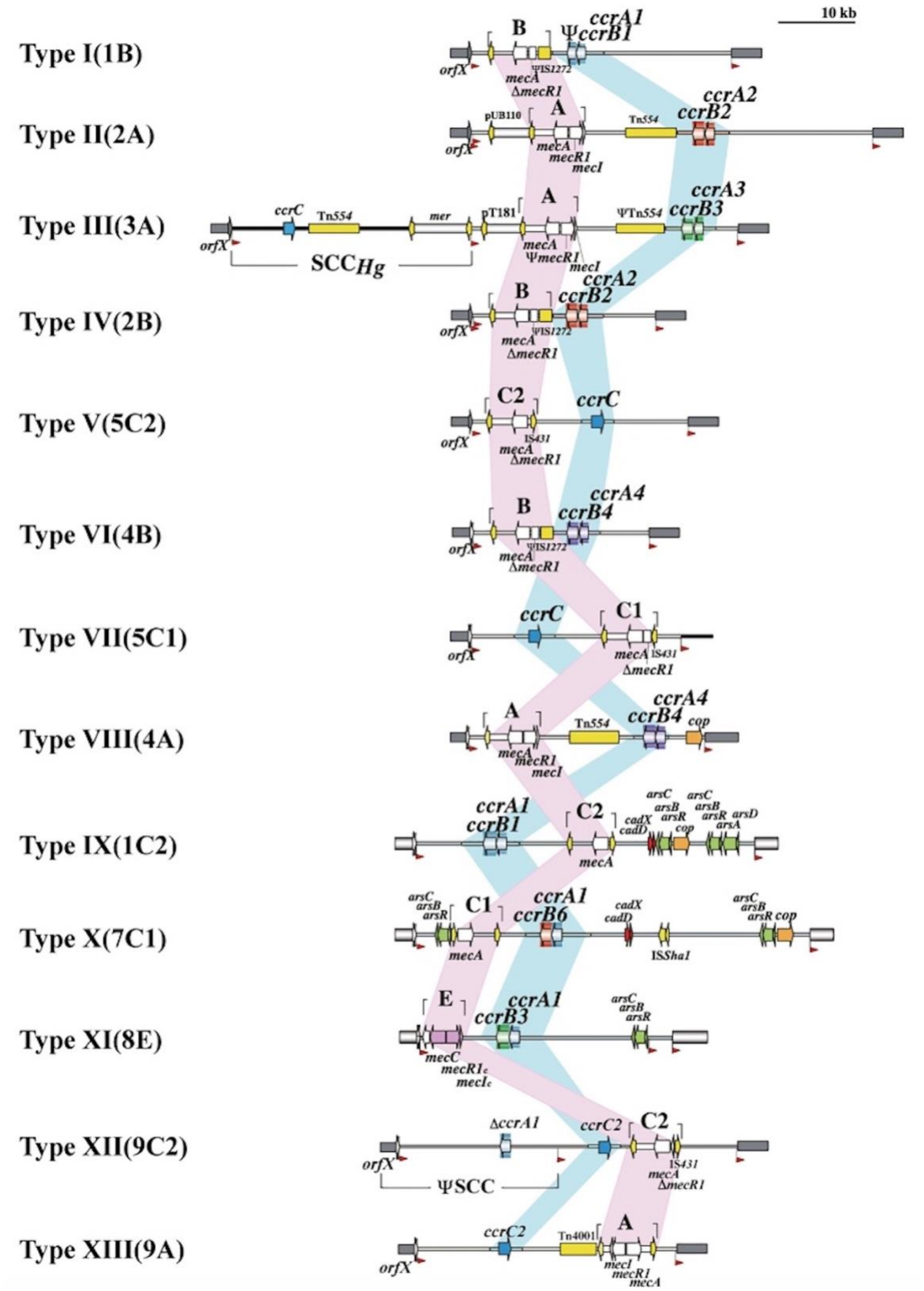


Figure 1.2 A schematic diagram showing the genetic organisation of the 13 SCC*mec* types identified in *S. aureus*, to date. The locations of the *mec* complexes are indicated by the pink belt. The locations of the *ccr* complexes are indicated by the blue belt. Adapted from Baig et al., 2018.

This formed SCC*mec*, which diversified in other CoNS species (e.g. *Staphylococcus epidermidis, Staphylococcus hominis* and *Staphylococcus haemolyticus*), and was eventually acquired by *S. aureus* via horizontal gene transfer (HGT) (Miragaia, 2018).

1.5 *Staphylococcus aureus* typing

Staphylococcus aureus is a heterogenous species with a clonal population structure. The majority of its genotypic variation therefore derives from point mutations, and recombination is a comparatively rare event (Enright et al., 2000). These characteristics render *S. aureus* an ideal candidate for typing. Bacterial typing is used to infer isolate relatedness and thus, forms the basis of outbreak investigations, local surveillance studies, global epidemiology investigations and phylogeny studies. The information gained from these investigations facilitates the development of more effective infection prevention and control (IPC) strategies, and provides valuable insight into the evolutionary dynamics of lineages or species. In the past, *S. aureus* typing has relied exclusively on phenotypic techniques such as antimicrobial resistance profiling, phage typing, biotyping and serotyping. However, as these traditional methods often lack reproducibility and offer only low-level discriminatory power, they have been largely replaced by molecular techniques. An overview of the most commonly used conventional molecular techniques for *S. aureus* typing, and/or those used during the present study, is provided below.

1.5.1 Pulsed-field gel electrophoresis (PFGE)

Emerging in the mid-1980s, PFGE was one of the earliest molecular typing methods developed, and subsequently became the "gold standard" for *S. aureus* typing during outbreaks (Goering, 2010; Tenover et al., 1995). The PFGE process involves the digestion of chromosomal DNA by rare-cutting restriction endonuclease enzymes, and the separation of the resulting fragments by PFGE. Unlike standard gel electrophoresis, the voltage gradient periodically alternates during PFGE, promoting the separation of large DNA molecules. The complex banding patterns generated are then analysed and compared using specialised software. Although considered highly sensitive, PFGE is labour-intensive and lacks inter-laboratory reproducibility. Acknowledgment of these limitations led to the development of standardised sequence-based typing methods, such as multilocus sequence typing (MLST), *S. aureus* protein A gene (*spa*) typing, and for MRSA specifically, direct repeat unit (*dru*) typing and SCC*mec* typing.

1.5.2 MLST

During MLST, ~450-bp internal fragments of seven unlinked housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*), are amplified by polymerase chain reaction (PCR) and sequenced via the Sanger method (Enright et al., 2000; Sanger et al., 1997). For each locus, distinct alleles are assigned a unique number, and every combination of alleles is assigned a different sequence type (ST), using the curated MLST website, https://pubmlst.org (formerly, http://www.mlst.net). The eBURST (Based Upon Related Sequence Types) algorithm can subsequently be used to assign STs to clonal complexes (CCs), which comprise genetically related strains. A typical CC is composed of a single predominant, or founding, genotype and its numerous less prevalent relatives. The CC model operates under the assumption that the founding genotype will gradually diversify as it becomes more common, and over time, mutations will occur in one of the seven MLST loci, leading to the emergence of a single locus variant (SLV). Eventually, the SLV will diversify further to become a double locus variant (DLV) of the founding genotype, and so on (Feil et al., 2004). Sequence types which do not share five of seven loci with any other ST are not associated with a CC and are termed "singleton" STs (Feil et al., 2004). Multilocus sequence typing has been particularly useful for assessing the population structure of *S. aureus* and identifying major global lineages (Fig. 1.3). However, as the seven MLST housekeeping genes are relatively stable, this technique does not provide the resolution necessary for outbreak investigations.

1.5.3 *spa* typing

The *S. aureus* genome includes loci harbouring stretches of repeated DNA motifs, the number of which varies between strains (Lindsay and Holden, 2006). These variable-number tandem-repeat (VNTR) regions can be exploited for typing purposes. The 3' end of the *spa* gene harbours a VNTR region, known as the X region, which comprises ~24 bp repeat units (Frénay et al., 1996). During *spa* typing, this region is amplified by PCR and sequenced via the Sanger method (Sanger et al., 1997). Online software (available at http://ridom.de/staphtype) is then used to assign a unique code to each new repeat variant and the strain is assigned a *spa* type based on its repeat succession. While *spa* typing provides less discriminatory power than PFGE, it is more discriminatory than MLST and involves the amplification and sequencing of just one locus (Shopsin et al., 1999). Furthermore, MLST STs/CCs can often be directly inferred from isolate *spa* types. These advantages have led to *spa* typing being widely adopted in national reference centres throughout Europe. However, *spa* typing only sometimes provides the discriminatory

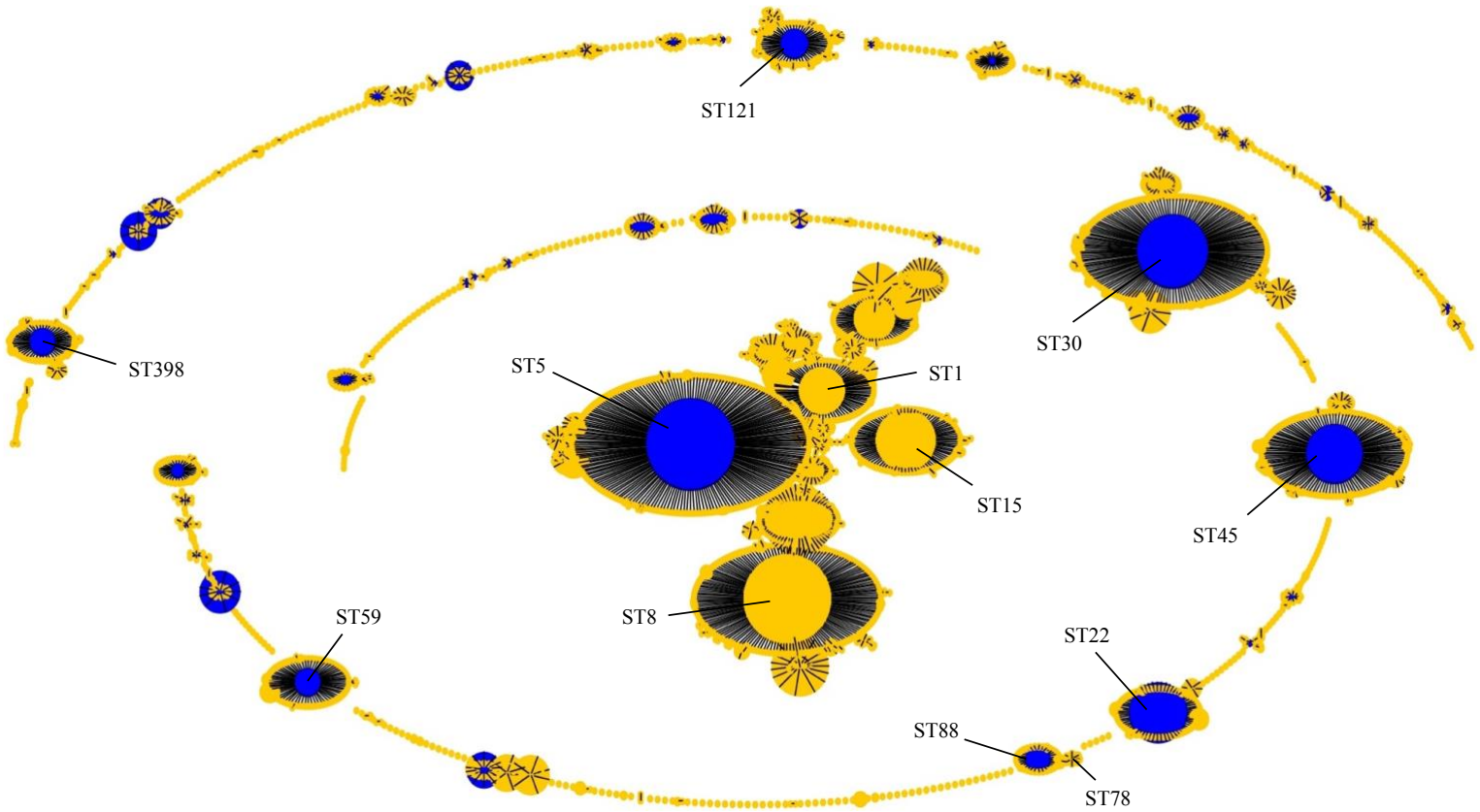


Figure 1.3 The population structure of the *S. aureus* as determined by conventional multilocus sequence typing (MLST). Each circle represents a different sequence type (ST). Circle size indicates the frequency of a particular ST within the database. Lines are drawn between all sequence types which share 6/7 alleles (single locus variants, SLVs). Sequence types which share at least 5/7 alleles are grouped into clusters termed clonal complexes (CCs). Blue circles within CCs represent 'founders', defined as the ST with the greatest number of SLVs. Examples of important lineages are indicated. This figure was constructed using eBURST v.3 (http://eburst.mlst.net) and all data from the *S. aureus* MLST database (http://saureus.mlst.net) as of November 2018.

power required to identify outbreaks and cannot provide insight into the dynamics of transmission.

1.5.4 *dru* typing

The *dru* region is a non-coding DNA segment that consists of 40-bp imperfect repeats. It is located between *mecA* and IS*431* in SCC*mec* (Goering et al., 2008). During *dru* typing, this region is amplified, sequenced and assigned a *dru* type using online software (http://www.dru-typing.org). While this technique is similar to *spa* typing, it cannot reliably differentiate between strains independently and can only be applied to MRSA (Shore et al., 2010). Its use is therefore not particularly widespread.

1.5.5 SCC*mec* typing

SCC*mec* typing involves determining the *mec* and *ccr* complex types of a MRSA isolate, while subtyping is performed by identifying relevant structural differences in the J regions. Numerous SCC*mec* typing and subtyping schemes have been developed, the majority of which involve multiplex PCRs (Chen et al., 2009; Kondo et al., 2007; Milheiric and Oliveira, 2007). No currently published PCR-based scheme encompasses all SCC*mec* types and subtypes identified in MRSA, to date. Accurate SCC*mec* assignment may therefore require the use of several multiplex PCRs, which can be expensive and time-consuming. Furthermore, typing schemes must be continuously updated to enable the identification of novel SCC*mec* types/subtypes.

1.5.6 DNA microarray profiling

The availability of the whole-genome sequences for an extensive range of *S. aureus* strains led to the development the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany). This kit consists of a high-throughput DNA microarray that detects the presence of genes/alleles encoding a broad range of typing markers, resistance determinants and virulence factors. Genotypic information of both clinical relevance (e.g. resistance and virulence genes) and epidemiological relevance (e.g. CCs/STs and basic SCC*mec* types) can therefore be obtained for a large number of isolates in a relatively short time frame (Monecke et al., 2008). Although DNA microarray profiling thoroughly characterises isolates and can accurately assign MRSA isolates to previously defined clones, it does not provide the discriminatory power required for outbreak investigations.

1.5.6.1 Enhanced SCCmec typing using DNA microarrays

A SCC*mec* subtyping DNA microarray has recently been trialled, which includes the targets for an extensive SCC*mec* subtyping scheme. Using publicly available reference sequences, this scheme defined 54 different SCC*mec* subtypes in *S. aureus*, based on differences in the J regions (Monecke et al., 2016). While this additional layer of typing can enable the differentiation of distinct MRSA clones with the same CC/ST and SCC*mec* type, it does not provide the resolution necessary to discriminate between closely related isolates.

1.5.7 Whole-genome analysis

Despite their value in recent decades, all of the sequence-based typing methods described above consider only small sections of the *S. aureus* genome and thus, provide limited resolution. In recent years, whole-genome sequencing (WGS)-based approaches have revolutionised bacterial typing by offering interlaboratory reproducibility and unprecedented discriminatory power. By employing these techniques, extensive information of both clinical (e.g. species identification, resistance and virulence potential) and epidemiological (e.g. relatedness to other isolates in the hospital/country) relevance is gained using a single protocol. Furthermore, WGS protocols are species-independent and thus, their routine use could enable the streamlining of operations in medical and surveillance laboratories. Although likely to reduce overall hospital expenditure (Mellmann et al., 2016), the set-up costs associated with implementing routine WGS have deterred many healthcare professionals from effecting this significant procedural adjustment. Furthermore, the challenges associated with data analysis and interpretation currently represent a major obstacle to hospital and reference laboratories, as procedures generally must be standardised in these settings. Therefore, although implemented/trialled in countries such as Denmark, Germany and France (Bartels et al., 2015; Durand et al., 2018; Joensen et al., 2014; Mellmann et al., 2016; Pinholt et al., 2017), the vast majority of clinical and surveillance laboratories worldwide do not currently use WGS-based typing methods. Further organism-specific WSG-based research is required in order to facilitate the widespread shift from conventional sequence-based typing to WGS-based typing. The technologies and main analysis methods associated with WGS-based typing are described in the following sections.

1.6 WGS

Sequencing platforms can be divided into three main categories or "generations". An overview of the most commonly used technologies from each of these generations, is provided below.

1.6.1 First generation sequencing

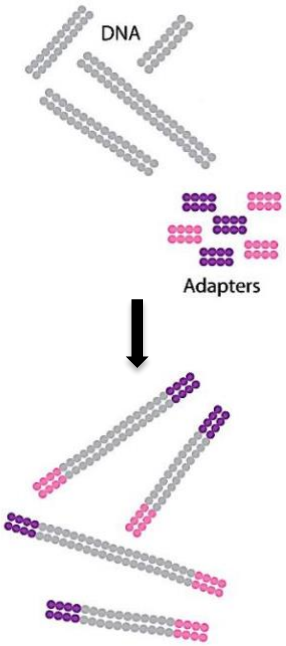
First generation sequencing refers to the Sanger and Maxam-Gilbert technologies, however, the former is the only of these methods still in use today (Maxam and Gilbert, 1977; Sanger et al., 1977, 1997). Sanger sequencing involves the addition of fluorescently labelled dideoxynucleotides triphosphates (ddNTPs) into four different (one for each ddNTP group) DNA extension reactions. This ensures termination of synthesis at every possible position and the generation of DNA fragments of all possible lengths (Sanger et al., 1997). The reaction products are electrophoresed, and a chromatogram is produced in which each coloured peak corresponds to a different nucleotide base, revealing the DNA sequence of the sample. Although highly accurate and cheaper than other sequencing methods, this technology is suited to short sequence lengths (<1000 bp), only.

1.6.2 Second Generation Sequencing/Next Generation Sequencing

The widespread application of WGS has been enabled by the development of Next Generation Sequencing (NGS) platforms, which emerged commercially in 2005 (Margulies et al., 2005). Although several NGS platforms (e.g. Roche 454, Ion Torrent and SOLiD) have been launched since 2005, they have been largely superseded by Illumina technology.

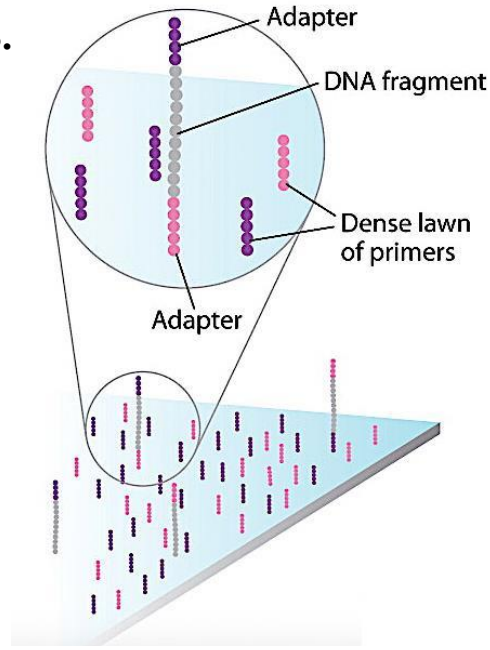
The Illumina sequencing process begins with library preparation. High molecular weight DNA is sheared to a specific size range and adapter sequences (including barcodes) are ligated to the fragmented ends (Fig. 1.4 [A]) (Mardis, 2008). The sample is then denatured and dispensed into a reagent cartridge, which is inserted into the sequencer. Inside the sequencer, the single stranded DNA fragments bind to the surface of a flow cell coated with primers complementary to the adapter sequences (Fig. 1.4 [B]) (Mardis, 2008). Each ligated fragment is repeatedly amplified (Fig. 1.4 [C] and [D]), and individual template clusters are produced on the surface of the flow cell (Mardis, 2008). Fluorescently labelled nucleotides are then incorporated into a new DNA strand that complements the template. In between each addition, a laser excites the newly incorporated nucleotide and a characteristic fluorescence is emitted (Fig. 1.4 [E]) (Mardis, 2008). This signal is recorded

A.



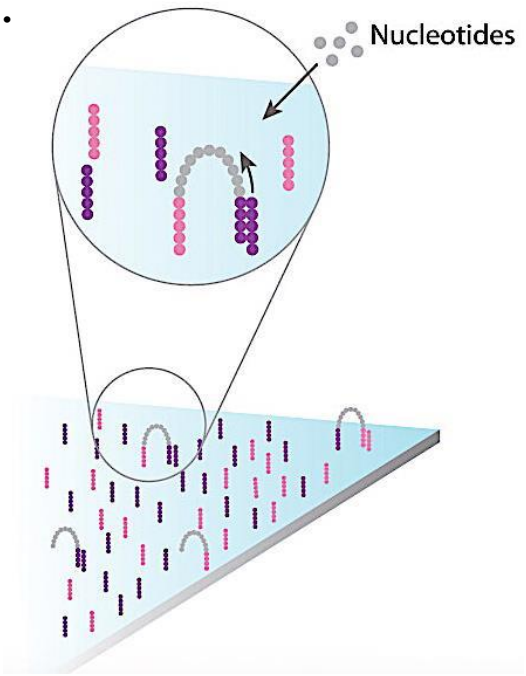
Adaptors are ligated to the fragmented input DNA.

B.



Adaptors bind to complementary primers on the surface of the flow cell.

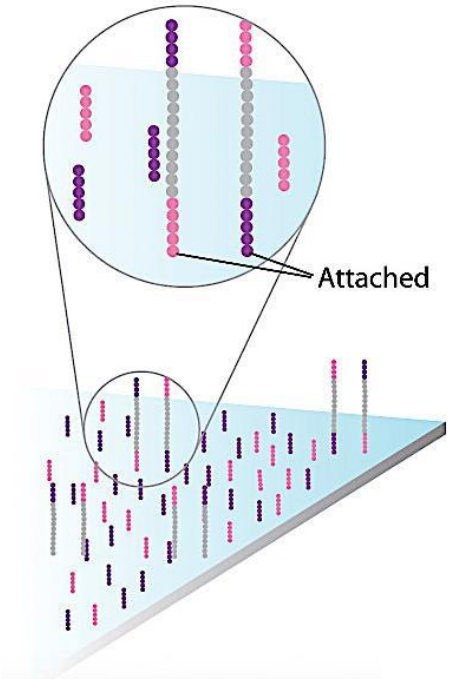
C.



The hybridised fragment folds over and binds a second adapter sequence.

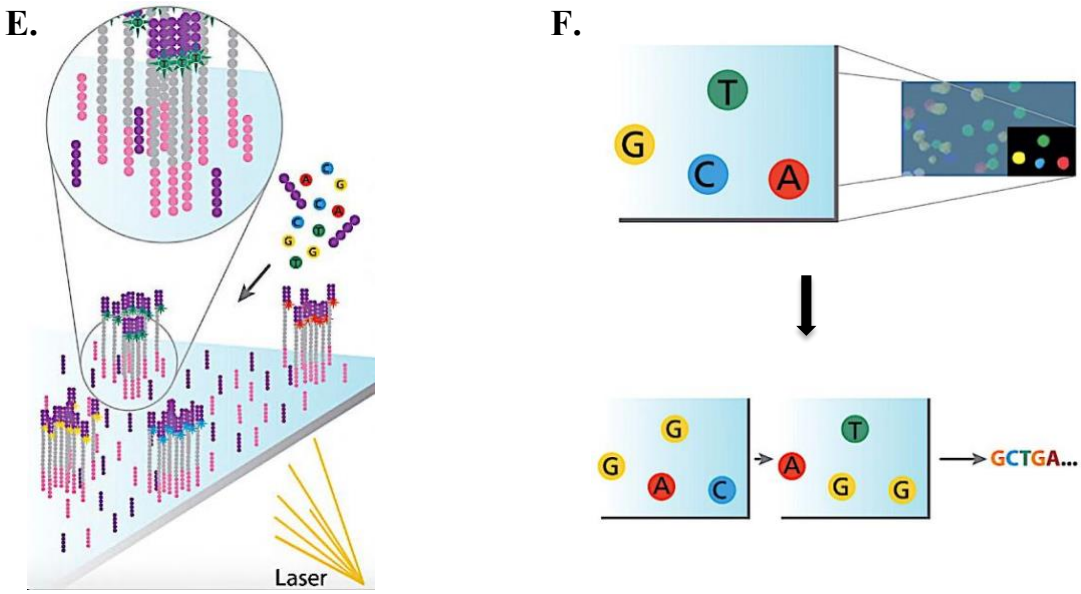
Polymerase incorporates nucleotides to build a double-stranded bridge.

D.



The double-stranded molecule is denatured, creating two single-stranded molecules.

The bridging process is continuously repeated and the reverse strands are washed away.



Fluorescently labelled nucleotides blocked at the 3'-end, compete for addition to the nucleotide chain.

A laser excites each nucleotide added.

A characteristic fluorescence, associated with the base added to the nucleotide chain, is emitted.

The 3'-end of the strand is unblocked, and the next nucleotide is incorporated into the chain.

The sequencer records all data generated to produce sequence reads.

Figure 1.4 A schematic representation of Illumina sequencing technology. Adapted from Mardis, 2008.

by the sequencer and single-end sequences read are generated (Fig. 1.4 [F]) (Mardis, 2008). Alternatively, the sequencing process can be repeated (from the opposite end of the template strand) to generate paired-end sequence reads and improve downstream analysis (Mardis, 2008). On Illumina systems, error rates in base calling are predicted by a quality scoring system, where a Q10 score indicates an error rate of 1 in 10 base calls, Q20 indicates an error rate of 1 in 100, Q30 indicates an error rate of 1 in 1000, Q40 indicates an error rate of 1 in 10,000, and Q50 indicates an error rate of 1 in 100,000. The quality of Illumina data is relatively high, although Q score decreases towards sequence read ends (Quainoo et al., 2017). Depending on the output required, Illumina sequencing runs take 4-56 h to complete.

The Illumina MiSeq system has been particularly widely used, to date. This is largely due to its more affordable set-up costs, shorter sequencing run times and longer read lengths, compared to other systems, while still maintaining high-quality data (Quainoo et al., 2017). Additionally, its lower output (0.5-15 Gb) compared to systems such as the NextSeq (16-120 Gb), makes the MiSeq particularly well-suited to microbial WGS (Quainoo et al., 2017). Although instrumental to genomic research thus far, Illumina sequencing produces short sequence reads (<300 bp) which must undergo extensive processing before data analysis can begin.

1.6.3 Third Generation Sequencing

There is considerable discussion about the characteristics that define a new generation of sequencing, particularly between the second and third generations (Heather and Chain, 2016; Mcginn and Gut, 2013). Here, third generation sequencing platforms are referred to as technologies capable of sequencing single molecules, therefore negating the need for an amplification step. Currently, the two major third generation sequencing platforms are Pacific Biosciences and Oxford Nanopore Technologies (ONT).

Pacific Biosciences operates a third-generation sequencing platform based on single-molecule real-time (SMRT) technology. During SMRT sequencing, the strand ends of the target DNA molecule are ligated by hairpin adaptors (Rhoads and Au, 2015). The sample is then loaded onto a SMRT cell containing microscopic chambers called zero-mode waveguides (ZMWs), which act as detection spaces during sequencing (Rhoads and Au, 2015). Inside the ZMW, the hairpin adapter binds to an immobilised DNA polymerase at the bottom of the chamber. Fluorescently labelled nucleotides are then added and

incorporated into a new DNA strand (Rhoads and Au, 2015). As the strand extends, the fluorescent labels are cleaved off and a characteristic light pulse is emitted. These pulses are detected by a laser beam and recorded in real time to determine the nucleotide sequence the target DNA (Rhoads and Au, 2015). Although SMRT sequencing technically has a higher error rate (11-15%) than NGS platforms, these errors are random and can therefore be largely discounted by the generation of a consensus sequence (Besser et al., 2018). The main advantage of SMRT technology compared to NGS, is its ability to produce read lengths of >20 kb (Quainoo et al., 2017). However, the high costs associated with SMRT technology have resulted in its being used largely to generate reference sequences against which short NGS reads can be aligned.

Oxford Nanopore Technologies, sometimes referred to as fourth generation sequencing, has become increasingly popular in recent years. This technology measures the changes in electric current caused by a single DNA molecule transiting through a pore, generating sequencing data in real-time (Mcginn and Gut, 2013). Although portable and inexpensive compared to other sequencing platforms, ONT currently has a high error rate (12-38%) and is therefore primarily used in combination with NGS platforms (Besser et al., 2018).

1.7 NGS-based *S. aureus* typing

The vast majority of WGS-based typing performed thus far has utilised NGS technologies and most frequently, Illumina platforms. Following an Illumina sequencing run, one (for single end reads) or two (for paired end reads) FASTQ files are generated for each sample included in the library. These files contain all sequence read and quality data associated with the sample. In order to extract useful genomic information from these files, several data analysis steps must be performed. These analyses are usually executed using either command line tools or commercial software packages. While the use of command line tools requires significant bioinformatic expertise, commercial packages operate on graphical user interfaces, and require little bioinformatics knowledge. Nonetheless, a basic understanding of the algorithms being applied to the data is recommended in order to avoid misinterpreting results (Quainoo et al., 2017). Currently, the most popular commercial analysis software suites are BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) and SeqSphere (Ridom GmbH, Münster, Germany). While both of these software packages offer extensive functionality, BioNumerics provides a particularly broad range of algorithms and parameter settings. Alternatively, web-based tools may be used to analyse

WGS data, although these are typically more limited in scope, slower and less reliable than both command line tools and commercial packages (Quainoo et al., 2017).

1.7.1 Assembly

The majority of downstream analyses require the assembly of sequence reads into contiguous sequences, known as contigs. While contigs represent the sequenced genome, it is impossible to produce a true copy of the original genome using NGS technology. This is largely due to the presence of repeat regions which are longer than the sequence reads, throughout the genome (Quainoo et al., 2017). Assembly becomes restricted in these regions, resulting in the occurrence of intermittent gaps of unknown sequence. While this limitation can be alleviated through the use of paired end sequencing, the genome sequence will always be split between numerous contigs (Quainoo et al., 2017). Velvet and SPAdes are the most frequently used short read assembly algorithms, however, SPAdes typically outperforms Velvet by generating fewer contigs (Bankevich et al., 2012; Zerbino, 2011). These algorithms are available as command line tools, web-based tools or as part of BioNumerics (Velvet and SPAdes) and/or SeqSphere (Velvet only).

1.7.2 Comparative genomics

Multiple different methodologies can be used to compare isolate genomes and ultimately, determine their relatedness. These approaches perform slightly different functions and exhibit varying levels of discriminatory power. The main comparative genomics approaches used, to date, include SNV analysis (also known as SNP analysis), core-genome MLST (cgMLST), whole-genome MLST (wgMLST) and core-genome SNV (cgSNV) analysis. Depending on the purpose of the study, these techniques may be used independently or in combination.

1.7.2.1 SNV analysis

Standard SNV analysis involves the use of a single reference genome to detect SNVs throughout the entire query genome(s). The sequence reads of the query genome are aligned to the reference using a mapping algorithm which allows for some variation between the reads and reference (Li and Durbin, 2010). A consensus query genome is then generated and SNVs can be identified between the sequences. Specific SNV filters can also be applied to ensure that all recorded variations represent true points mutations, and not assembly errors or recombination events. This technique offers exceptionally high resolution and has been used during *S. aureus* transmission studies, retrospective outbreak

investigations and surveillance (Azarian et al., 2016; Bartels et al., 2015; Price et al., 2017). A disadvantage of this technique, however, is the requirement of a suitable reference genome. If the query and reference genomes are not closely related, lineage-specific regions could be absent from the reference and thus, excluded from the analysis (Quainoo et al., 2017). It may therefore be appropriate to use a study-specific reference, particularly during outbreak investigations (Roisin et al., 2016).

1.7.2.2 cgMLST

Core-genome MLST is an extension of conventional MLST, which vastly increases resolution without the requirement of a reference genome. This method excludes the accessory genome, which may be appropriate during long-term studies, or when analysing isolates recovered from different regions/environments (Mellmann et al., 2017). The *S. aureus* cgMLST scheme includes 1,861 loci, seven of which form the traditional MLST scheme (Leopold et al., 2014). This well-established scheme has been successfully employed for the real-time surveillance of multidrug-resistant (MDR) bacteria in a large German hospital, on a trial basis (Mellmann et al., 2016). Although providing lower resolution than SNV analysis, cgMLST offers a standardised method via which isolates from all lineages can be compared and thus, may prove particularly useful to reference laboratories in the future.

1.7.2.3 wgMLST

Whole-genome MLST is an extension of cgMLST that includes accessory genome loci, therefore further increasing resolution. This method is suitable for identifying closely related isolates in a local setting and has been used to investigate both MRSA and MSSA outbreaks (Roisin et al., 2016; Sabat et al., 2017; Weterings et al., 2017). Although the accessory genome includes MGEs that can be horizontally acquired, a closely related group of isolates will typically harbour MGEs that were each introduced into the population on a single occasion, and then inherited vertically (Planet et al., 2017). Excluding the accessory genome can therefore eliminate important evidence during a comparative analysis of closely related isolates.

1.7.2.4 cgSNV analysis

A disadvantage of the extended MLST schemes described above is that the number of SNVs occurring at each locus is not considered. While investigating closely related isolates, this is unlikely to significantly skew the data as one allelic difference will likely

correspond to one SNV. While comparing isolates from different lineages, however, a single allelic difference could correspond to multiple SNVs. It may therefore be appropriate to employ cgSNV analysis during genomic comparisons that include distantly related isolates. Core-genome SNV analysis involves the concatenation of core genome loci and the generation of a multiple sequence alignment from which SNVs can be identified. This method therefore eliminates the requirement of a reference genome, while providing nucleotide-level resolution. Although not yet widely applied to *S. aureus*, this approach is used during the surveillance of vancomycin-resistant enterococci (Pinholt et al., 2017).

1.7.2.5 Phylogeny trees

The datasets generated from the above analyses can be visually represented using a range of phylogenetic trees including unweighted pair group method with arithmetic (UPGMA) trees, minimum spanning trees (MSTs), neighbour joining tree (NJTs), maximum parsimony trees (MPTs) and/or maximum likelihood trees (MLTs). These trees can be broadly categorised as distance-based (involving the use of a distance matrix to determine the similarity of isolates; UPGMA, MST, NJT) or character-based (involving the use of sequence data to determine the number of substitutions per site; MPT, MLT) (Brinkman, 2001). Although the true accuracy of these algorithms in estimating phylogeny cannot be definitively determined, they serve as useful tools during WGS data interpretation (Brinkman, 2001). While character-based methods are suited to SNV analysis only, distance-based algorithms provide a simple model to facilitate the comparison of isolate genomes based on SNVs or allelic differences. Two of the most widely used distance-based trees are MSTs and NJTs. The MST algorithm directly connects nodes by the shortest possible distance (Krusal, 1956) and in certain instances, can therefore be used to infer the most likely chain of pathogen transmission. The NJT algorithm also aims to minimise branch length but does not directly connect nodes (Saitou and Nei, 1987).

1.7.2.6 Data interpretation

There are significant challenges associated with WGS data interpretation. Recently, it has been suggested that *S. aureus* exhibiting ≤ 24 cgMLST/wgMLST allelic differences or ≤ 15 SNVs may be considered closely related (Schürch et al., 2018). However, this is a highly complex issue and all thresholds must be treated as guidelines, only. The

consideration of any available epidemiological data can also facilitate sequence data interpretation (Schürch et al., 2018).

1.8 The evolution and molecular epidemiology of MRSA

Different lineages of *S. aureus* have acquired SCC*mec* on multiple occasions (Miragaia, 2018; Robinson and Enright, 2003; Tsubakishita et al., 2010). Many different MRSA clones therefore exist worldwide, some of which have gained endemic or pandemic status (Monecke et al., 2011). In 2002, an international nomenclature scheme was developed whereby clones could be defined by their ST and SCC*mec* type e.g. ST1-MRSA-IV (Enright et al., 2002). Although practical, this system is also limited as *S. aureus* of the same ST may acquire the same SCC*mec* type on different occasions. In these instances, clones are usually differentiated by an additional characteristic, such as the presence/absence of the genes encoding PVL (Monecke et al., 2011). However, as more MRSA clones emerge and existing clones diversify, the current MRSA nomenclature system may become less viable. Distinct MRSA clones have emerged in healthcare settings, the community and among animals.

1.8.1 Healthcare-associated MRSA (HA-MRSA) clones

MRSA was initially associated exclusively with hospitals, and patients infected with MRSA typically exhibited specific risk factors (e.g. older age, immunocompromised state, recent surgery) (Otter and French, 2010). The majority of HA-MRSA clones derived from five different lineages; CC5, CC8, CC22, CC30 and CC45 (Chambers and Deleo, 2009; Otter and French, 2010). The CC5 and CC8 lineages are the most prevalent worldwide, and each include multiple MRSA clones (e.g. CC5, ST5-MRSA-II; CC8, ST8-MRSA-IV) which have spread in many regions. The CC22 lineage, which includes the ST22-MRSA-IV clone, is also widespread, but particularly common in Europe and Australia. The CC30 lineage includes at least two major MRSA clones (ST36-MRSA-IV and ST36-MRSA-II), one of which (ST36-MRSA-IV) was previously particularly common in the UK. Finally, the CC45 lineage includes the ST45-MRSA-IV clone, which is common in Europe and the USA (Chambers and Deleo, 2009; Stefani et al., 2012). These MRSA clones are often resistant to additional antibiotic classes, apart from β-lactams, and are therefore particularly suited to the hospital environment (Bal et al., 2016).

1.8.2 Community-associated MRSA (CA-MRSA) clones

MRSA of community origin was first detected in rural Australia in the late 1980s (Udo et al., 1993), and gained increased attention after emerging in the USA in the early 1990s (Herold et al., 1998). Unlike HA-MRSA, CA-MRSA has traditionally been associated with the infection of otherwise healthy individuals and risk factors such as playing team sports, living/working in crowded areas, and having a low socioeconomic status (David and Daum, 2010). Although capable of causing serious invasive infections, CA-MRSA are more often associated with SSTIs (DeLeo et al., 2010). Examples of major CA-MRSA clones identified to date are listed in Table 1.4. These clones typically harbour SCC*mec* types IV or V and are not usually MDR (David and Daum, 2010). It has been suggested that the carriage of these smaller SCC*mec* types and the absence of additional resistance genes has increased the fitness of CA-MRSA, compared to HA-MRSA (Otto, 2013). Furthermore, the observation that CA-MRSA are capable of infecting otherwise healthy individuals has led to the suggestion that these clones exhibit enhanced virulence. This has been supported by studies which demonstrate that CA-MRSA typically express increased levels of PSMs (Wang et al., 2007). Additionally, some CA-MRSA clones harbour virulence determinants that are generally absent from HA-MRSA, such as PVL (e.g. USA400; ST1-MRSA-IV), or the arginine catabolic mobile element (which facilities colonisation; e.g. USA300; ST8-MRSA-IV) (Chambers and Deleo, 2009).

1.8.3 MRSA clones in animals

MRSA of animal origin were first recovered from dairy cows with mastitis in Belgium, in 1971 (Devriese et al., 1972). Multiple livestock-associated (LA)-MRSA clones have since emerged, deriving from *S. aureus* lineages associated either primarily with animals (e.g. CC130), or with both humans and animals (e.g. CC1) (Fitzgerald, 2012). In Europe, the predominant LA-MRSA lineage is CC398, which is particularly prevalent in pigs (Bal et al., 2016). Livestock-associated MRSA typically harbour SCC*mec* types IV or V, although types IX, X and XII are also associated with livestock (Feßler et al., 2012; Kadlec et al., 2011; Li et al., 2011; Wu et al., 2015). Interestingly, it has been suggested that animals may have been the source of *mecC*, associated with SCC*mec* XI, as *mecC*-positive isolates generally harbour an intact *hlb* gene (García-álvarez et al., 2011; Monecke et al., 2013b; Shore et al., 2011). Furthermore, the majority of *mecC*-positive MRSA isolates identified, to date, have been recovered from animals (Paterson et al., 2014). Resistance to non-β-lactam antibiotics is also common among LA-MRSA, likely due to their extensive use in the livestock industry (Allen and Stanton, 2014). In addition to livestock, MRSA has also

Table 1.4 Examples of prevalent community-associated MRSA clones

Lineage	Clone	Comment	Main associated region(s)	Reference
CC1	PVL-positive ST1-MRSA-IV	Also known as USA400 and MW2; first CA-MRSA clone identified in the USA; was displaced by USA300	USA, Canada	Herold et al., 1998
	PVL-negative ST1-MRSA-IV	Also known as WA MRSA-1; first CA-MRSA clone identified; first detected in Aboriginal communities in Western Australia	Australia	Coombs et al., 2011
	ST772-MRSA-V	PVL-positive; also known as the Bengal Bay clone; becoming increasingly prevalent in Europe	India, Bangladesh	Monecke et al., 2013
CC8	ST8-MRSA-IV	PVL-positive, also known as USA300; predominant CA-MRSA clone in the USA; has achieved pandemic spread	USA, Canada	Nimmo, 2012
CC30	ST30-MRSA-IV	PVL-positive; also known as the SWP clone; has achieved pandemic spread	South America, Oceania, East Asia	Skov et al., 2012
CC59	ST59-MRSA-V	PVL-positive; also known as the Taiwan clone	East Asia, Australia	Monecke et al., 2011
CC80	ST80-MRSA-IV	PVL-positive; also known as the European clone; possibly originated in North Africa; predominant CA-MRSA clone in Europe	Europe, North Africa, The Middle East	Tenover, 2010
CC88	ST88-MRSA-IV	PVL-negative; known to be endemic only in Africa	Africa	Breurec et al., 2011
	ST78-MRSA-IV	PVL-negative; also known as WA MRSA-2	Australia	Coombs et al., 2012

Abbreviations: CC, clonal complex, PVL, Panton-Valentine leukocidin; ST, sequence type; SWP, South West Pacific; WA, Western Australia

been recovered from wild animals and companion animals (Fitzgerald, 2012; Monecke et al., 2016a). Unlike LA-MRSA, studies demonstrate that MRSA recovered from companion animals are often of human origin (Baptiste et al., 2005; Fitzgerald, 2012; Vincze et al., 2014).

1.8.4 The blurring of traditional boundaries

In the past, MRSA clones were generally detected in the environment in which they initially arose, only. In the literature, the acronyms HA-MRSA, CA-MRSA and LA-MRSA are therefore sometimes used in an epidemiological sense. For example, patients who develop MRSA infections in hospital more than 48 h post admission are often said to have contracted HA-MRSA, regardless of the clone type (David and Daum, 2010). In the present study, these acronyms are used in a genotypic sense, only. In recent years, CA-MRSA have become increasingly prevalent in healthcare settings and mathematical models suggest that CA-MRSA will eventually replace HA-MRSA (Agata et al., 2009; Bal et al., 2016; Brennan et al., 2012). Furthermore, CA-MRSA clones are gradually acquiring additional resistance determinants, increasing their fitness in the hospital environment (Bal et al., 2016; Brennan et al., 2012; Wang et al., 2012). Similarly, studies indicate that humans often acquire LA-MRSA, likely via direct contact and/or the food chain (Smith, 2015). This is of particular concern as LA-MRSA often exhibit resistance to antibiotics also used in human medicine.

1.9 MRSA in Ireland

1.9.1 Surveillance

In 1998, The European Antimicrobial Resistance Surveillance Network (EARS-Net) was established to monitor antimicrobial resistance among several bacterial pathogens causing bloodstream infections (BSIs) in Europe, including *S. aureus*. Currently, all 28 EU countries, Norway and Iceland submit data to EARS-Net on a quarterly basis. In Ireland, all EARS-Net isolates are collected by the Irish National MRSA Reference Laboratory (NMRSARL). The NMRSARL was opened in 2002, in St. James's Hospital, Dublin, with the primary aim of assisting clinical microbiology laboratories in accurately typing their isolates. Upon submission to the NMRSARL, all isolates undergo *spa* typing and susceptibility testing against a range of antimicrobial agents. An in-house multiplex PCR for *mecA*, *mecC*, *nuc* (to positively identify *S. aureus*) and *lukF/S-pvl* is also performed. Although functioning largely as a typing facility, the NMRSARL does not currently have the resources to perform high resolution WGS-based analyses on a routine basis.

1.9.2 Prevalence

According to EARS-Net reports, the rate of MRSA among *S. aureus* BSIs in Ireland peaked in 2006, reaching 42% (592/1412) (EARS-Net, 2006). This figure decreased steadily for the following ten years, falling to 14.7% (172/1170) by 2016 and reflecting overall European trends (EARS-Net, 2016). Interestingly, for reasons that remain unclear, the incidence of MRSA among *S. aureus* BSIs in Ireland increased again in 2017, rising to 16.3% (192/1175) (EARS-Net, 2017). Ireland currently ranks mid-level among the remainder of Europe in this regard, with countries such as Denmark and Sweden exhibiting MRSA rates of 1-5%, in 2017, while others such as Greece and Italy reported rates of 25-50% (Fig. 1.5) (EARS-Net, 2017). It is important to note, however, that these data relate to *S. aureus* recovered from BSIs, only. Therefore, while prevalence of MRSA among hospital-acquired *S. aureus* infections in Ireland/Europe may have decreased between 2006 and 2016, MRSA likely became increasingly prevalent in the community during this time, as suggested by surveillance studies and in line with global trends (David and Daum, 2010; Kinnevey et al., 2014; Otter and French, 2010; Rossney et al., 2007; Shore et al., 2014).

1.9.3 Molecular epidemiology

MRSA was first reported in Ireland in 1971, and has been endemic in Irish hospitals since the 1980s (Coleman et al., 1985; Hone and Keane, 1974; Rossney and Keane, 2002; Shore et al., 2010). Retrospective surveillance revealed that the predominant MRSA clone in Ireland changed three times between 1971 and 2002 (Shore et al., 2005). In the 1970s and early 1980s, ST250-MRSA-I/I-*pls* was predominant. This clone was displaced in the mid to late 1980s by ST239-MRSA-III/III-pI258/Tn*554*, which was then replaced by ST8-MRSA-IIA-IIE in the 1990s (Shore et al., 2005). Since 2002, the ST22-MRSA-IV clone has predominated in Irish hospitals, accounting for 70-80% of all MRSA BSI isolates recovered per annum (NMRSARL, 2017). However, an extensive range of other MRSA clones has also been identified in Ireland. Indeed, 38 different *spa* types and 25 SCC*mec* types/subtypes were identified among 88 non-ST22-MRSA-IV isolates identified at the NMRSARL between 2000 and 2012 (Kinnevey et al., 2014). Furthermore, a considerable increase in the prevalence of CA-MRSA was reported in Irish hospitals and the community, between 2002 and 2011 (Shore et al., 2014). Interestingly, international travel has repeatedly been identified as a contributory factor to the high level of MRSA diversity in Ireland (Brennan et al., 2012; Rossney et al., 2007; Shore et al., 2014). Consistent surveillance of these irregular strains is important in order to identify reservoirs of

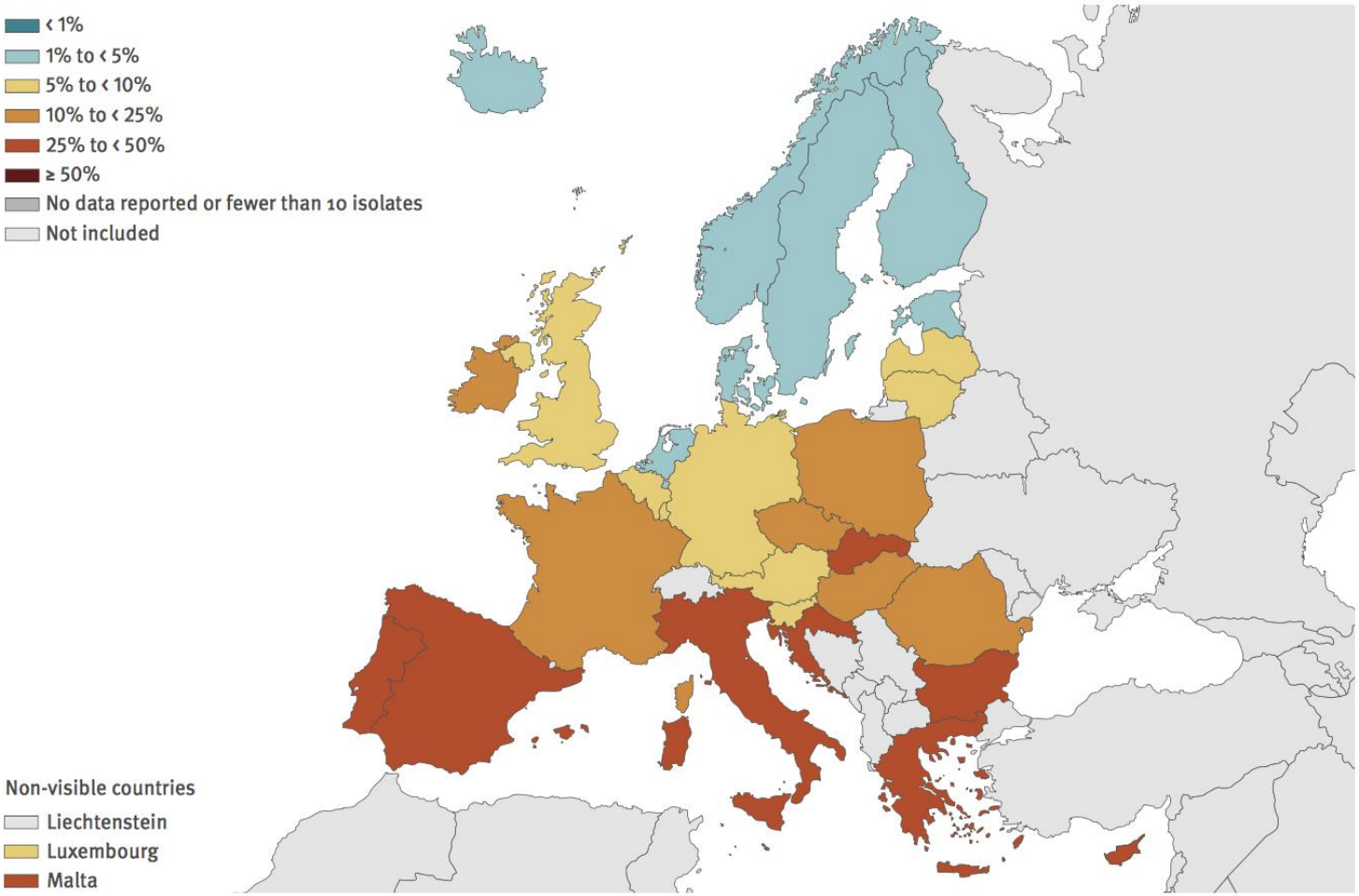


Figure 1.5 The prevalence of MRSA among *S. aureus* causing bloodstream infections in Europe, in 2017. Adapted from EARS-Net, 2017.

resistance and virulence-associated genes, and to detect emerging clones with the potential for widespread dissemination.

1.9.4 Screening/decolonisation in hospitals

In Irish hospitals, patients are screened for MRSA upon admission only if they exhibit specific risk factors for HA-MRSA carriage (e.g. recent hospitalisation, employment as a HCW) or are at increased risk of developing an infection (e.g. have non-intact skin, are undergoing elective surgery) (Irish Department of Health, 2013). All patients are screened upon admission into an intensive care unit (ICU) or neonatal ICU (NICU), and weekly thereafter. Routine patient screening is also performed during an outbreak (Irish Department of Health, 2013). If a patient is identified as MRSA-positive, decolonisation is considered only if the patient is at risk of developing an infection, or if uncontrolled MRSA transmission persists on the ward, despite the implementation of other IPC measures (Irish Department of Health, 2013). Nasal and body decolonisation are typically performed using 2% mupirocin and 4% chlorhexidine, respectively. Healthcare worker screening is recommended upon identification of an infection cluster, however, this is not mandatory (Irish Department of Health, 2013).

1.9.5 Treatment guidelines

Depending on the infection type, Irish guidelines generally recommend doxycycline (a member of the tetracycline antibiotic class), glycopeptides (such as vancomycin), linezolid (an oxazolidinone) or daptomycin (a cyclic lipopeptide), to treat MRSA infections (Irish Department of Health, 2013). It is advised that macrolides (e.g. erythromycin) and fluoroquinolones (e.g. ciprofloxacin) be avoided, due to the frequency with which MRSA (particularly the predominant and endemic ST22-MRSA-IV clone (Irish NMRSARL, 2016)) exhibit resistance to these agents (Irish Department of Health, 2013).

1.10 Aims

1. While the pandemic ST22-MRSA-IV clone predominates in Irish hospitals, PVL-negative ST1-MRSA-IV (of the CA CC1 lineage) have become increasingly prevalent in both hospitals and the community in recent years. This is partially due to the occurrence of a protracted outbreak of high-level mupirocin-resistant (MupR) PVL-negative ST1-MRSA-IV in a large Dublin hospital between 2013 and 2016. The first part of the present study aimed to employ WGS to (i) characterise the *ileS2*-encoding plasmid conferring mupirocin resistance to these outbreak isolates, (ii) confirm/dispute the establishment of a

single clonal outbreak and (iii) investigate the relatedness of the outbreak isolates to other PVL-negative ST1-MRSA-IV recovered in Ireland in order to determine whether the outbreak strain spread beyond a single hospital.

2. The results from the first part of this study demonstrated that the PVL-negative ST1-MRSA-IV clone becoming increasingly prevalent in Ireland exhibited multidrug resistance and thus, did not fully match the description of the well-defined CA PVL-negative ST1-MRSA-IV clone known as Western Australia (WA) MRSA-1. The second part of this study therefore aimed to investigate the origin of the MDR PVL-negative ST1-MRSA-IV clone, using WGS, in order to determine whether it constitutes a MDR sub-clone of WA MRSA-1 or a distinct PVL-negative CC1-MRSA-IV clone, yet to be characterised.

3. Between 2009 and 2017, 22 MRSA isolates exhibiting two different *spa* types associated with the CA CC88-MRSA lineage, were identified in the NICU of an Irish hospital. The third part of this study aimed to use WGS to (i) investigate the relatedness of these isolates and identify putative transmission events, (ii) investigate the relatedness of these isolates to other CC88 MRSA identified at the NMRSARL during the study period and (iii) investigate the geographic origin of the CC88-MRSA isolates by comparing them to international CC88 MRSA isolates.

4. Although HCWs have been definitively linked to *S. aureus* outbreaks in recent years, routine HCW screening for MRSA is not performed in Ireland. The primary aim of the fourth part of this study was to determine whether HCWs constitute a significant source of *S. aureus* in patients in non-outbreak scenarios, using WGS. Additionally, as the prevalence of MRSA among *S. aureus* BSIs is decreasing in Ireland, the number of MSSA causing invasive infections is rising. The secondary aim of the fourth part of this study was to investigate the population of *S. aureus* (MRSA and MSSA) in a large Dublin hospital, using WGS.

Chapter 2

General Materials and Methods

2.1 Bacterial isolates

As detailed in Table 2.1, 425 *S. aureus* isolates were investigated during the present study. This included MRSA (*n* = 176) and MSSA (*n* = 196) isolates recovered in Ireland, and MRSA (*n* = 43) and MSSA (*n* = 10) isolates recovered internationally.

2.2 General microbiological methods

2.2.1 Isolate storage and bacterial culture

Isolates were stored in Microbank cryogenic bead vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C. Unless otherwise stated, isolates were cultured on Trypticase Soy Agar (TSA; Oxoid, Basingstoke, UK) or Columbia Blood agar (CBA; Fannin Ltd., Dublin, Ireland). Isolates were cultured by removing a single bead from the storage vial using sterile forceps, inoculating the appropriate media plate using a sterile inoculating loop, and incubating the plate overnight in a static incubator at 37°C.

2.2.2 Chemicals, water and buffers

All chemicals used were of analytical or molecular biology grade and were purchased from Merck Ireland Ltd. (Co. Wicklow, Ireland), unless otherwise stated. Water ultra-purified using the Milli Q Biocel system (Millipore Ireland, Co. Cork, Ireland) was used for the preparation of buffers and other chemical solutions. Tris-borate/EDTA (TBE) was used for agarose gel preparation and as a gel electrophoresis buffer. TBE was prepared to a stock concentration of 5x using 0.45 M Trizma base, 0.45 M boric acid and 0.01 M EDTA, pH 8, and diluted to a working concentration of 0.5x. Unless otherwise stated, Molecular Biology Grade Water (Merck) was used in all PCR reactions, and DNA dilutions and elutions.

2.3 Isolate identification and antimicrobial susceptibility testing

2.3.1 Processing of clinical and screening samples

In Chapters 3 and 6, some or all of the *S. aureus*/MRSA investigated were isolated from clinical swabs, as part of this study. Sa*Select* or/and MRSA*Select* chromogenic agar plates (Bio-Rad, Dublin, Ireland) were lawned with patient swabs, as specified per chapter. The plates were incubated for 48 h at 37°C. Presumptive *S. aureus* and/or MRSA colonies were identified as those exhibiting a strong pink colour (Fig. 2.1). These colonies were purified by subculturing onto TSA. The Pastorex Staph-Plus Kit (Bio-Rad) was then used to confirm/dispute their identity as *S. aureus*/MRSA. The Pastorex Staph-Plus Kit consists of

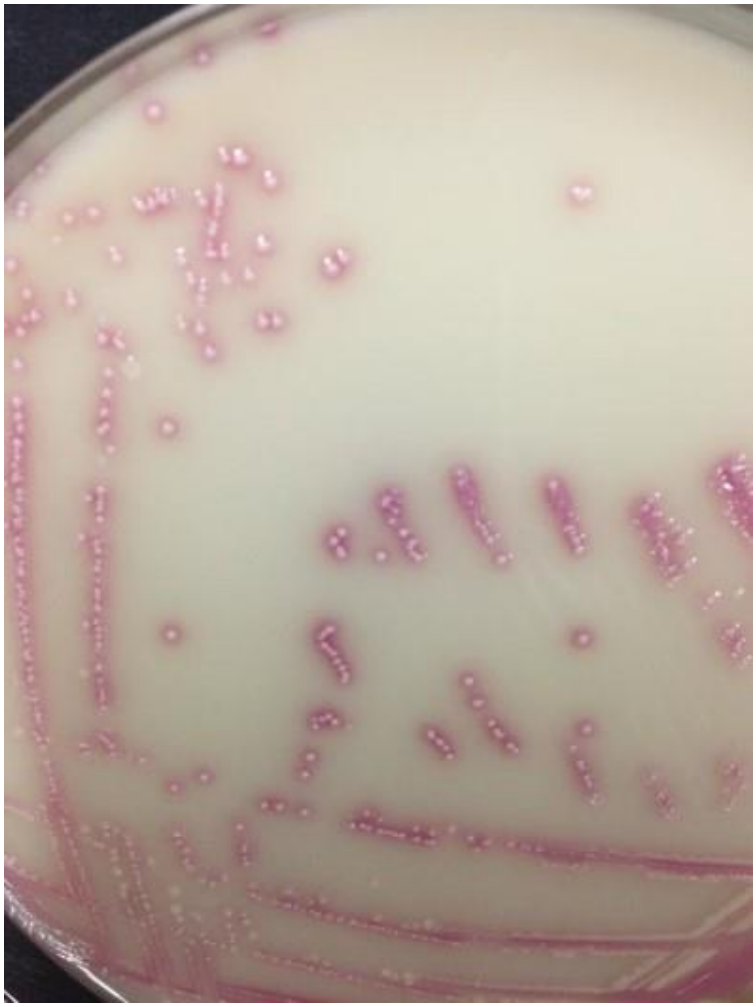


Figure 2.1 Photograph showing *S. aureus* colonies growing on Sa*Select* agar. *Staphylococcus aureus* colonies exhibit a strong pink colour on Sa*Selec*t chromogenic agar (BioRad, Dublin, Ireland) following incubation for 24 h at 37^{o}C.

Table 2.1 Details associated with the 425 *S. aureus* isolates investigated during the present study

Chapter	Title of substudy	Number of isolates		Years of isolation	Countries of isolation
		MRSA	MSSA		
3	The recent emergence and spread of high-level mupirocin-resistant ST1-MRSA-IV-t127 in Ireland	89	0	2013-2016	Ireland
4	The identification and characterisation of an emerging novel multidrug-resistant ST1-MRSA-IV-t127 clone which may have originated in South-Eastern Europe	167	20	2004-2018	Germany, Ireland, Romania, UAE,
5	Intra-hospital, inter-hospital and intercontinental spread of ST78-MRSA-IV from two neonatal intensive care unit outbreaks	43	0	2001-2017	Australia, Egypt, France, Germany, Ireland, Tanzania
6	The transmission of *Staphylococcus aureus* between healthcare workers, patients and the environment, in a large Irish hospital	9	186	2017	Ireland

[a]This included the 89 isolates investigated in Chapter 3.

an agglutination assay that simultaneously detects clumping factor, staphylococcal protein A and capsular polysaccharides, using latex particles sensitised with human fibrinogen and monoclonal antibodies.

2.3.2 Identification of isolates as *S. aureus*

Unless otherwise stated, all Irish isolates were identified/confirmed as *S. aureus* at the NMRSARL using the tube coagulase test, which detects the presence of the staphylocoagulase protein (Rossney et al., 1990). All international isolates were identified as *S. aureus* either at Abbott (Alere Technologies GmbH [Jena, Germany]) or the Institute for Medical Microbiology and Hygiene, Technical University of Dresden (Dresden, Germany), using the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry system (Vitek, bioMérieux, Marcy l'Etoile, France) (Dubois et al., 2012), according to the manufacturer's instructions.

2.3.3 Identification of *S. aureus* as MRSA or MSSA

Unless otherwise stated, all Irish isolates were identified/confirmed as MRSA at the NMRSARL using 30-µg cefoxitin disks (Oxoid, Basingstoke, England) in accordance with European Committee of Antimicrobial Susceptibility Testing (EUCAST) methodology and interpretive criteria (EUCAST, 2018). All international isolates were identified as methicillin-resistant either at Abbott (Alere Technologies GmbH) or the Institute for Medical Microbiology and Hygiene, Technical University of Dresden, using the automated VITEK 1 or VITEK 2 systems (bioMérieux, Nuertingen, Germany), according to the manufacturer's instructions.

2.3.4 Antimicrobial susceptibility testing

All isolates investigated in Chapters 3, 5 and 6 underwent antimicrobial susceptibility testing at the NMRSARL. The susceptibility of isolates was determined by disk diffusion against a panel of 20 antimicrobial agents (in addition to cefoxitin) using EUCAST methodology, and previously described reference strains and interpretative criteria (EUCAST, 2018; McManus et al., 2015). The antimicrobial agents tested and disk concentrations used are detailed in Table 2.2. The most recently applied breakpoints are also included in Table 2.2, although a selection of these breakpoints varied slightly during the time period in which antimicrobial susceptibility testing was performed for this study (i.e. between 2004 and 2018). MSSA were considered MDR if they exhibited resistance to

Table 2.2 Antimicrobial agents and breakpoints used for susceptibility testing during the present study

Antimicrobial agent	Disk concentration (µg/disk)	Zone breakpoints (mm)[a]			Reference
		S ≥	I[b]	R <	
Amikacin	30	18	None	16	(EUCAST, 2018)
Ampicillin	10	29	None	28	(CLSI, 2018)
Cefoxitin	30	22	None	22	(EUCAST, 2018)
Chloramphenicol	30	18	None	18	(EUCAST, 2018)
Ciprofloxacin	5	21	None	21	(EUCAST, 2018)
Clindamycin	2	22	None	19	(EUCAST, 2018)
Erythromycin	15	21	None	18	(EUCAST, 2018)
Fusidic acid	10	24	None	24	(EUCAST, 2018)
Gentamicin	10	18	None	18	(EUCAST, 2018)
Kanamycin	30	18	14-17	13	(CLSI, 2018)
Linezolid	10	21	None	21	(EUCAST, 2018)
Mupirocin	200	30	None	18	(EUCAST, 2018)
Neomycin	30	18	16-17	15	(Rossney et al., 2007)
Rifampicin	5	26	None	23	(EUCAST, 2018)
Spectinomycin	500	13	14-19	20	(Rossney et al., 2007)
Streptomycin	25	16	14-15	13	(Rossney et al., 2007)
Sulphonamide	300	17	13-16	12	(CLSI, 2018)
Tetracycline	30	22	None	19	(EUCAST, 2018)
Tobramycin	10	18	None	18	(EUCAST, 2018)
Trimethoprim	5	17	None	14	(EUCAST, 2018)
Vancomycin	30	15	None	14	(CLSI, 2018)

[a]Zones of growth inhibition were recorded in mm and interpreted as resistant (R), intermediate (I), or susceptible (S), according to the guidelines referenced. [b]None, no intermediate breakpoint indicated in guidelines.

three different antimicrobial classes, while MRSA were considered MDR if they exhibited resistance to three different classes, in addition to β-lactams.

2.4 Conventional molecular methods

2.4.1 Extraction of genomic DNA

DNA extractions were performed using either the DNeasy Blood and Tissue Kit (Qiagen, England, UK). Isolates were cultured on TSA or CBA, as described in section 2.2.1. For each isolate, a single colony was lawned onto a fresh CBA plate, using a sterile inoculating loop, and incubated overnight at 37°C. Lysis buffer was prepared one of two ways: (i) using 1x Tris-EDTA buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA, pH 8), lysostaphin (0.2 mg/ml; Merck) and lysozyme (0.02 mg/ml; Merck), a 250 µl volume of which was used per sample or (ii) using the Buffer A1 and Lysis enhancer A2 supplied in the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH]), a 200 µl volume of which was used per sample. Approximately 2.5cm^2 of the lawned culture growth was mixed with the lysis buffer in an Eppendorf tube (Eppendorf, Hamburg, Germany). This mixture was incubated for 2-3 h with shaking at 200 rpm in an orbital incubator. To degrade proteins and nucleases, 25 µl of proteinase K (20 µg/ml) and 200 µl of AL buffer (both supplied with the Qiagen DNeasy Blood and Tissue Kit) were added to the lysate and the Eppendorf tube was incubated for 30 min at 70°C. DNA was isolated from the lysate using the mini spin columns (with silica-gel membranes that selectively bind DNA), collection tubes and wash buffers supplied with the Qiagen DNeasy Blood and Tissue Kit, according to the manufacturer's instructions. Purified DNA was eluted into 50 µl (for DNA microarray profiling) or 150 µl (for WGS or PCR) of molecular biology-grade water in a fresh Eppendorf tube. Samples were stored at -20°C.

2.4.2 Quality assurance, concentration and dilution of genomic DNA

The Nanodrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA) was used to determine the DNA concentration and indicate the purity of samples produced as described in section 2.4.1. For DNA microarray profiling, samples were concentrated by heating for 30 min at 70°C, with the lids of the Eppendorf tubes open and subsequently, diluted to a concentration of 0.5-1.5 ng/µl. For WGS, all samples were diluted to a concentration of ~0.2 ng/µl using the Qubit Fluorometer 1.0 or 3.0 (ThermoFisher, Dublin, Ireland), according to the manufacturer's instructions. The DNA extraction protocol was repeated if WGS samples did not exhibit 260/280 nm and 230/260 nm absorbance ratios of

approximately 1-8-2.0 and 2.0-2.2, respectively, indicating the presence of proteins or other contaminants.

2.4.3 *spa* typing

spa typing was performed at the NMRSARL. Genomic DNA was extracted using InstaGene matrix (BioRad, München, Germany), according to the manufacturer's instructions. The variable X region in the *spa* gene of each isolate underwent PCR amplification using the primers and thermal cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; http://www.seqnet.org). The Ridom StaphType software package version 1.5 (Ridom GmbH, Würzburg, Germany) was used for *spa* sequence analysis and *spa* type assignment.

2.5 Whole-genome sequencing

2.5.1 Library preparation

The Nextera XT Library Preparation and Nextera XT Index (24 indexes) Kits (Illumina, Eindhoven, the Netherlands) were used to prepare DNA for WGS. In Chapters 3, 4 and 5, libraries were scaled to exhibit approximately 100x coverage, i.e. each sequencing run included 12 isolates. In Chapter 6, libraries were scaled to exhibit approximately 50x coverage, i.e. each sequencing run included 24 isolates.

2.5.1.1 Tagmentation

For each DNA sample, 5 µl of Tagment DNA Buffer (Illumina), 2.5 µl of input DNA (diluted as described in section 2.4.2) and 2.5 µl of Amplicon Tagment Mix (Illumina) were mixed in a 0.2 ml PCR tube. This mixture was incubated at 55ºC for 5 min (Fig. 2.2). To ensure timely tagmentation cessation, 2.5 µl of Neutralising Tagment Buffer (Illumina) was immediately added to each sample and all samples were incubated for 5 min at room temperature.

2.5.1.2 Amplification of libraries

Each tagmented DNA sample was mixed with 7.5 µl of Nextera PCR Master Mix (Illumina) and 2.5 µl each of index adapters i5 and i7 (Illumina). Unique index adapter combinations were used for samples to be included in the same MiSeq (Illumina) sequencing reaction. The resulting mixtures underwent amplification using a Kyratec Thermocycler model SC200 (Kyratec, Mansfield, Australia) or G-storm GSI Thermocycler (G-Storm, Somerset, England) and the following thermal cycling conditions: 72ºC for 3

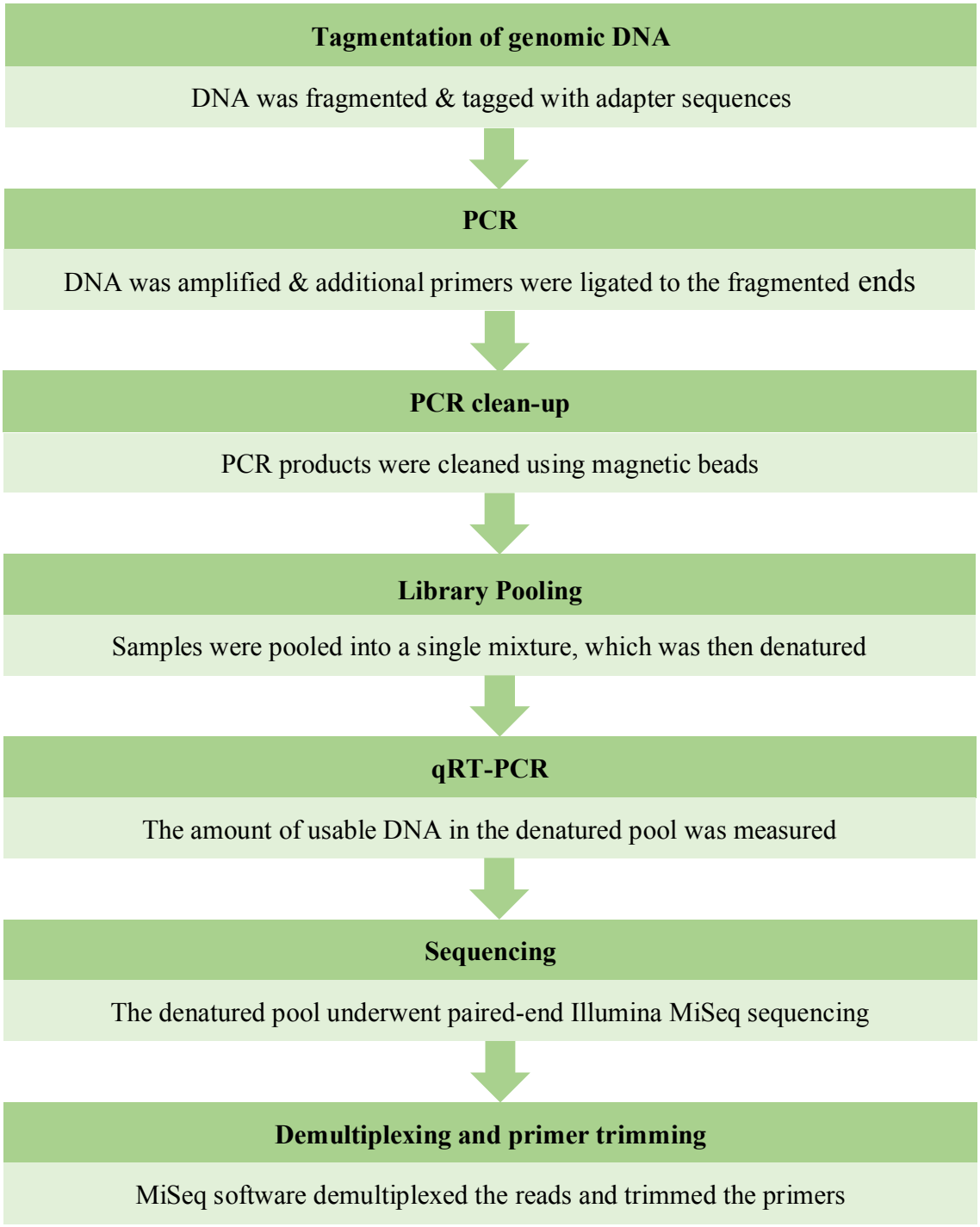


Figure 2.2 A simplified summary flow diagram of the library preparation and sequencing procedure used during the present study.

min, 95ºC for 30 s, 12 cycles of 95ºC for 10 s, 55ºC for 30 s and 72ºC for 30 s, 72ºC for 5 min (Fig. 2.2).

2.5.1.3 PCR clean-up

The PCR clean-up was performed using Solid Phase Reversible Immobilization (SPRI) beads. SPRI beads are magnetite-surrounded polystyrene beads coated in carboxyl molecules that can reversibly bind DNA (Deangelis et al., 1995). Each PCR product was mixed with 12.5 µl of AMPure XP beads (Beckman Coulter, Clare, Ireland) and transferred into a Thermowell 96-well polycarbonate PCR plate (Corning, Flintshire, UK), which was subsequently incubated at room temperature for 5 min. The 96-well plate was then placed on a Magnetic Stand-96 (Thermo Fisher Scientific, Dublin, Ireland) for 2 min. During this time period, the AMPure XP beads aggregated at the side of each well, beside the magnet. The supernatant was then carefully removed without disrupting the beads and each well was washed three times with 180 µl of freshly prepared 80% (v/v) ethanol. The beads were allowed to air-dry for 15 min at room temperature. The 96-well plate was then removed from the magnetic stand and the contents of each well were resuspended in 26.5 µl of Resuspension Buffer (Illumina). The 96-well plate was placed back onto the magnetic stand for 2 min. During this time period, the DNA detached from the AMPure XP beads and remained in solution while the magnetic beads aggregated at the side of each well. Eluted library volumes of 25 µl were then transferred from each well into individual 0.2 ml tubes. Libraries were stored at -20ºC. Libraries were stored from a maximum of seven days (Fig. 2.2).

2.5.1.4 Library quantification, pooling and denaturation

The DNA concentration of each library was measured using the Qubit Fluorometer 1.0 or 3.0 (Thermo Fisher), according to the manufacturer's instructions. An equal amount of DNA from each library (in a minimum volume of 1 µl) was pooled into a single 0.2 ml tube. Libraries exhibiting disproportionately low DNA concentrations, indicative of extensive sample loss, were excluded from the pool. Library preparation was repeated for these samples. The pooled library was mixed with freshly diluted 0.1N NaOH at a ratio of 1:1. The resulting mixture was incubated at room temperature for 5 min to allow denaturation to occur. The denatured pool was mixed with Library Normalization Storage Buffer 1 (Illumina) at a ratio of 1:1. In Chapters 4, 5 and 6, the diluted denatured pool was stored for a maximum of 24 h (Fig. 2.2).

2.5.1.5 Quantitative real-time PCR (qRT-PCR)

The denatured pool underwent quantification using the SYBR Fast Rox Low Kit (Kapa Biosystems, London, England) for Illumina platforms, according to the manufacturer's instructions. This kit contains six DNA standards and a Master Mix, the latter of which includes probes that are complementary to the adapter sequences in the Nextera XT Index Kit (Illumina). Reaction mixtures were prepared in 20 µl volumes which included 12.4 µl of the Master Mix and 4 µl of the denatured pool (previously diluted at a ratio of 1:1000). These mixtures were organised in a MicroAmp Optical 96-well Reaction Plate (Thermo Fisher) and covered with MicroAmp Optical Adhesive Film (Thermo Fisher). The reaction was performed using an Applied Biosystems 7500 Real Time PCR system (Thermo Fisher) and the following thermal cycling conditions: initial denaturation at 95ºC for 5 min, 35 cycles of 95ºC for 30 s and 60ºC for 45 s, and melt curve analysis (which functions to identify any adapter-dimer carry over that may result in the calculation of an inflated library concentration) at 65-95ºC. Applied Biosystems 7500 Software v.2.0.6 (Thermo Fisher) was used to analyse the raw data generated (Fig. 2.2).

2.5.1.6 Final denaturation, MiSeq loading and sequence run quality assurance

The denatured library was diluted to a concentration of 12 pM using Library Normalization Storage Buffer 1 (Illumina). Immediately prior to MiSeq loading, the diluted denatured pool was incubated at 96ºC for 2 min to ensure that the sample was fully denatured. All isolates underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina; Fig. 2.2). Upon MiSeq loading, the demultiplexing and primer trimming were functions were applied (Fig. 2.2). Following each sequencing run, the quality of the data generated was assured by cluster density and Q30 assessment, according to the manufacturer's (Illumina) instructions.

2.6 WGS data processing and analysis

2.6.1 Data exportation and read trimming

Following each sequencing reaction, the forward and reverse FASTQ files for each isolate were exported from the MiSeq computer (Fig. 2.3). The sequence reads were then trimmed using BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). The default quality, structural and read length trimming algorithms were applied. The quality trimming algorithm removed all base calls below 10% of the average quality. The structural trimming algorithm removed low quality read ends using a rolling average quality

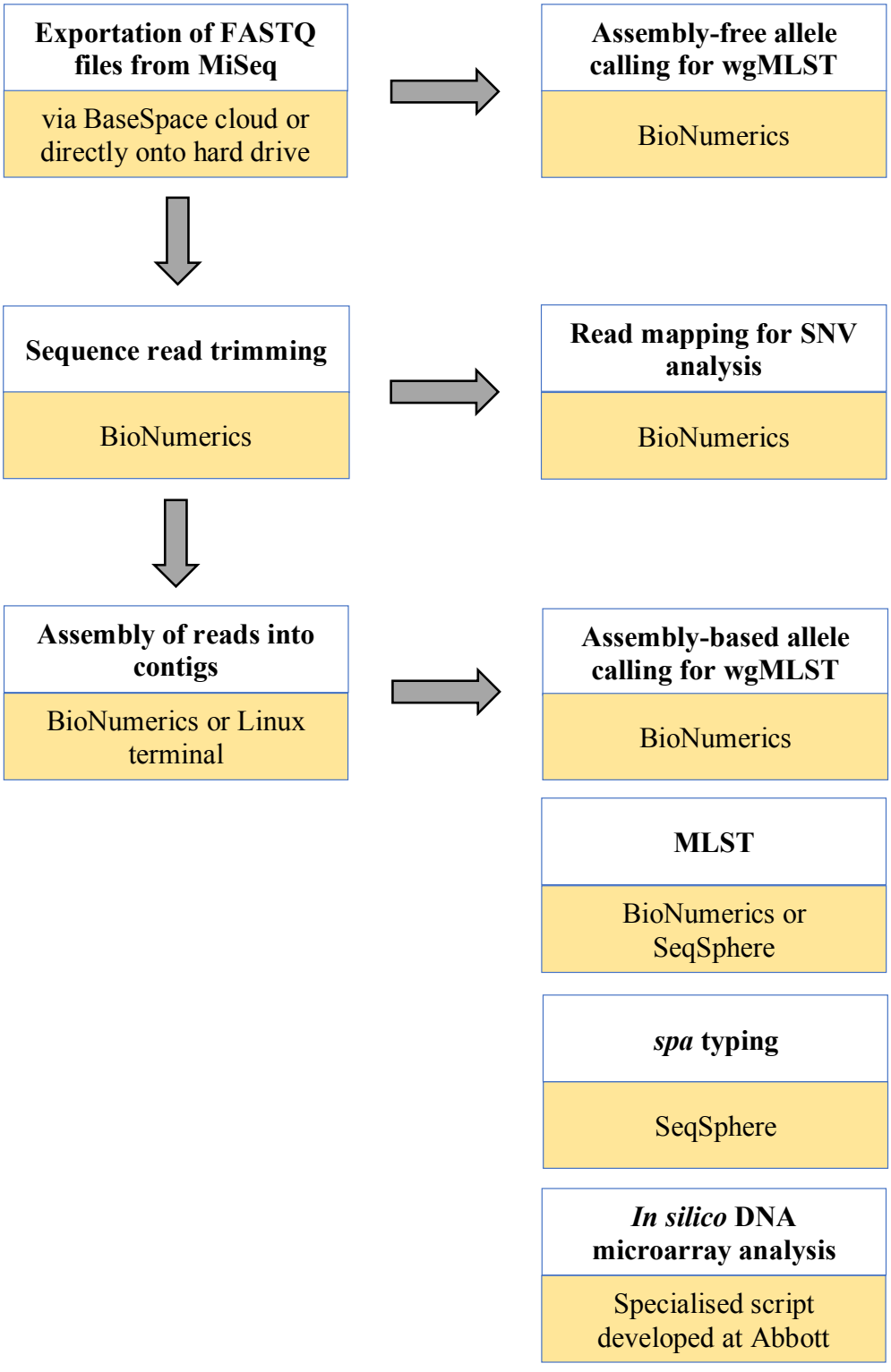


Figure 2.3 A simplified flow diagram summary of the main WGS data processing and analysis methods used during the present study. Abbreviations: SNV, single nucleotide variation; wgMLST, whole-genome multilocus sequence typing.

threshold of 25% of the average quality. The read length algorithm removed reads below 10% of the average read length (Fig. 2.3).

2.6.2 *de novo* assembly

In Chapters 4, 5 and 6, isolate genomes were assembled using the SPAdes genome assembler v3.7.1 (Bankevich et al., 2012). SPAdes automatically identifies the optimal assembly parameters for each assembly. Contigs were stored as FASTA files. Contigs under 1000 bp were considered unreliable and were therefore removed (Fig. 2.3).

2.6.3 MLST

WGS-based MLST was performed on contigs using Ridom SeqSphere+ version 4.1 (Ridom GmbH) which connects to the publicly available MLST scheme provided by PubMLST (https://pubmlst.org; Fig. 2.3). Newly identified MLST alleles and profiles were submitted to PubMLST for incorporation into the PubMLST system.

2.6.4 *spa* typing

WGS-based *spa* typing was performed on SPAdes-assembled contigs using Ridom SeqSphere+ version 4.1 (Ridom GmbH; Fig. 2.3) (Bletz et al., 2015). If *in silico spa* typing failed, isolates underwent real-life *spa* typing as described in section 2.4.3

2.6.5 Genotyping and SCC*mec* subtyping using *in silico* DNA microarrays

In silico genotyping involved the detection of 338 target sequences which correspond to the allelic variants of 187 different genes, in each isolate contig set. These genes included the species marker, virulence-associated, antimicrobial resistance and SCC-associated marker genes that ultimately determined the DNA microarray profile of an isolate. This scheme was based on that developed for the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH]), with which *in vitro* DNA microarray profiling can be performed. Details of the relevant alleles and probes have been previously described (Monecke et al., 2008). The SCC*mec* subtyping array targeted an additional 83 distinct sequences that are variably present in the SCC*mec* element and which form the basis of a recently described system that distinguishes between 54 different SCC*mec* subtypes (Monecke et al., 2016). Virtual DNA microarray hybridisation patterns were generated using a specialised script developed in Abbott (Alere Technologies GmbH), as previously described (Monecke et al., 2016b). SPAdes-assembled contigs were searched for probes (Fig. 2.3). The number of mismatches between the probe and target sequence identified determined the strength of

the virtual hybridisation signal. Perfect matches yielded the maximum strength signal, one mismatch yielded an attenuated maximum strength signal, two mismatches yielded half the maximum signal, three mismatches yielded a weak signal and four or more mismatches yielded no signal. Processed data were exported to Microsoft Excel v15.40 for analysis.

2.6.6 Selection of international comparator isolates based on *in silico* DNA microarrays

All international isolates included in this study were selected from the in-house *S. aureus* strain collections at Abbott (Alere Technologies GmbH) and the Institute for Medical Microbiology and Hygiene, Technical University of Dresden. This joint strain collection includes approximately 25,000 *S. aureus* isolates recovered from humans and animals worldwide, a selection of which have been previously reported (Monecke *et al.*, 2008, 2011, 2016). The DNA microarray profiles and/or SCC*mec* subtypes of all isolates in this collection are stored in a Excel large database. These genotypic data were generated using either the the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH]) or the *in silico* genotyping and SCC*mec* subtyping DNA microarrays described in section 2.6.5. In Chapters 4 and 5, the DNA microarray profiles and/or SCC*mec* subtypes of the Irish isolates were compared to those in this extensive database. International isolates exhibiting either similar or dissimilar (as specified per chapter) DNA microarray profiles and/or SCC*mec* subtypes to the Irish isolates were then selected for further investigation using WGS.

2.6.7 SNV analysis

BioNumerics v7.6 (Applied Maths) was used for SNV analysis. Trimmed sequence read sets were mapped against the contigs of a study-specific reference isolate using the Applied Maths Mapper tool, a custom implementation of the Burrows-Wheeler Aligner (Fig. 2.3) (Li and Durbin, 2010). The default base correction parameters were used during read mapping. Specifically, a single base threshold of 0.75 was applied, meaning that the most frequently called base at a given position constituted at least 75% of all base calls at that position in order to be called in the consensus sequence. A double base threshold of 0.85 was applied, meaning that the two most frequently called bases at a given position constituted at least 85% of all base calls at that position in order to be called in the consensus sequence. These positions are denoted with the International Union of Pure and Applied Chemistry (IUPAC) code for 2-fold degenerated positions (R: A/G; M: C/A; S: C/G; Y: C/T; W: A/T; K: G/T). A triple base threshold of 0.95 was applied, meaning that

the three most frequently called bases constituted at least 95% of the base calls to be considered the three possible bases at a certain position in the consensus sequence. These positions are denoted with the IUPAC code for 3-fold degenerated positions (V: A/C/G; H: A/C/T; D: A/G/T; B: C/G/T). Double base calling was only applicable for positions that did not fulfil the criterion for single base calling and triple base calling was only applicable for positions that did not fulfil the criteria for single or double base calling. A gap threshold of 0.5 was applied, meaning that at least 50% of bases were called for that position to be considered in the consensus sequence. The SNV filters used are specified per chapter.

2.6.8 wgMLST analysis

The BioNumerics v7.6 (Applied Maths) wgMLST plug-in was used to perform wgMLST analysis. This plug-in connects to a wgMLST scheme for *S. aureus* that consists of 3,904 loci, 1,861 of which constitute the cgMLST scheme previously defined by Leopold *et al.* (Leopold et al., 2014). This scheme was created by including all core and accessory genome loci identified in the 42 *S. aureus* genomes available in GenBank as for June 2014, as described in detail previously (Roisin et al., 2016). In order to ensure that all relevant alleles present were detected, two different algorithms were used to generate a consensus wgMLST profile for each isolate. The first method determined locus presence/absence and allelic identity using an assembly-free k-mer approach (Fig. 2.3). This computationally-favourable process operates by identifying of all possible sub-sequences (of length k) per raw sequence read and includes an in-built base call quality check. The default k-mer length of 35 was applied. Only loci with a minimum total coverage of 5x, including 1x coverage in both directions, were retained for consideration in the consensus wgMLST profile. The second, assembly-based method, used a BLAST approach to detect alleles in contigs (Fig. 2.3) (Altschul et al., 1990). The default minimum sequence similarity of 75% was applied and gapped alignments were allowed. Loci for which more than one allelic variant was detected in a single sample were excluded from the consensus wgMLST profile of that sample.

2.6.9 Quality assurance of WGS data

The quality of the sequence read sets, *de novo* assemblies, and assembly-free and assembly-based allele calls, were assessed using the quality statistics window in BioNumerics v7.6 (Applied Maths). Sequence reads were assessed based on their average quality score. Assemblies were assessed based on their N50 (i.e. the minimum contig

length required to cover 50% of the genome), average coverage and the number of contigs produced. WGS was repeated for samples in which less than 95% of core-genome loci were identified.

2.6.10 Phylogenetic tree generation

In Chapters 3, 5 and 6, MSTs were generated using distance matrices based on MLST, wgMLST, cgMLST and/or SNV data, as specified per chapter. In Chapter 4, NJTs were generated using distances matrices based on cgSNV analysis. All MSTs were generated in BioNumerics v7.6 (Applied Maths) with permutation resampling (1000 replicates).

Chapter 3

The recent emergence and spread of high-level mupirocin-resistant ST1-MRSA-IV-t127 in Ireland

3.1 Introduction

Mupirocin, also known as pseudomonic acid A, is an antibiotic produced by the bacterium *Pseudomonas fluorescens* (Uretsky et al., 1971). This small molecule inhibits bacterial protein synthesis by binding bacterial isoleucyl-tRNA synthetase (encoded by *ileS*), and is highly active against staphylococci, streptococci and certain Gram-negative bacteria (e.g. *Neisseria gonorrhoeae*) (Poovelikunnel et al., 2015; Sutherland et al., 1985). Mupirocin, which is rapidly inactivated following systemic administration, has been used topically in hospitals since the early 1980s (Dacre et al., 1983; Poovelikunnel et al., 2015). In the many countries in which infection prevention strategies include *S. aureus* decolonisation, mupirocin ointment (usually 2% w/v) is applied to the anterior nares two or three times daily, for five to seven consecutive days (Poovelikunnel et al., 2015). Previous studies have detailed the excellent efficacy of mupirocin for short-term *S. aureus* eradication and its moderate efficacy for medium/long-term *S. aureus* eradication (Fritz et al., 2012; Van Rijen et al., 2008). In addition to decolonisation, mupirocin is used prophylactically at catheter exit sites and for the treatment of SSTIs in both hospitals and the community (Bernardini et al., 1996; Sutherland et al., 1985). Furthermore, mupirocin is often used in veterinary medicine to treat canine *Staphylococcus pseudintermedius* infections (Godbeer et al., 2014).

Mupirocin resistance is generally categorised as either low-level (mupirocin minimum inhibitory concentration [MIC] 2-128 mg/L) or high-level (mupirocin MIC > 256 mg/L), both of which emerged in staphylococci in the late 1980s (EUCAST, 2018; Cookson, 1989; Patel et al., 2009; Rahman et al., 1987). High-level resistance generally occurs due to the acquisition of an alternate and plasmid-borne *ileS* gene, termed *ileS2* (*mupA*) (Hodgson et al., 1994; Udo et al., 2001). High-level mupirocin resistance has been identified in all the major pandemic HA-MRSA lineages (i.e. CC5, CC8, CC22, CC30, CC45) and previous studies indicate that global prevalence rates in MRSA range from 1-63% (Pérez-Roth et al., 2006; Poovelikunnel et al., 2015). Coagulase-negative staphylococci, such as *S. epidermidis* and *S. haemolyticus*, have been identified as important reservoirs for *ileS2* in *S. aureus* (Hetem et al., 2014; Rossi et al., 2016). For example, a 2012 study set in a Dutch hospital detailed the association between mupirocin use and an increase in CoNS exhibiting resistance to mupirocin and other antimicrobials (Bathoorn et al., 2012). Indeed, mupirocin usage has been shown to co-select for MGEs conferring resistance to unrelated antimicrobials (Carter et al., 2018). To date, 10 different

Table 3.1 The ten publicly available *ileS2*-encoding plasmid sequences previously characterised in detail

Plasmid name	Organism	Size (kb)	Plasmid family/families	Additional antimicrobial resistance gene(s)	*tra* locus	Experimental method(s)	GenBank accession number
pPR9	*S. aureus*	41.7	pSK41/pGO1	None	Present	Shotgun sequencing	NC_013653
pV030-8	*S. aureus*	39.0	pSK41/pGO1	None	Present	Unknown	NC_010279
pUSA03	*S. aureus*	37.1	pSK41/pGO1	*erm*(C)	Present	Shotgun sequencing	NC_007792
pGO400	*S. aureus*	~34.0	pSK41/pGO1	None	Present	Shotgun sequencing	FM207042
pT15G-1	*S. lugdunensis*	42.3	pSK41/pGO1	*aacA-aphD*, *aadD*, *qacC*	Present	Illumina sequencing	KU882681
pK93G	*S. lugdunensis*	25.7	pSK41/pGO1	*aacA-aphD*	Absent	Illumina and SMRT sequencing	KU882682
pT33G-1	*S. lugdunensis*	46.4	pSK41/pGO1 pT33G	*aacA-aphD*, *cadX*, *qacA*, *tet*(K)	Absent	Illumina sequencing and PCR gap closure	KU882683
pT8G	*S. lugdunensis*	31.8	pT33G	*aacA-aphD*, *aadD*, *cadX*, *lnu*(A), *qacA*, *tet*(K)	Absent	Illumina sequencing and PCR gap closure	KU882684
pT20G	*S. lugdunensis*	37.4	pT20G pT33G	*aadD*, *cadX*, *lnu*(A), *tet*(K)	Absent	Illumina sequencing and PCR gap closure	KU882685
pT63N	*S. lugdunensis*	40.6	pSP01 pT20G	*aadK*	Absent	Illumina sequencing and PCR gap closure	KU882686

Abbreviations: SMRT, single molecule real-time.

ileS2-encoding plasmids have been characterised in detail (summarised in Table 3.1). Plasmids encoding *ileS2* are often conjugative, and of the 10 *ileS2*-encoding characterised to date, five encode the *tra* locus (Rahman et al., 1989). In 2011, a second high-level mupirocin resistance-conferring gene designated *ileS3* (*mupB*) was identified in three MRSA isolates in Canada (Seah et al., 2012). Low-level mupirocin resistance, however, usually arises from a point mutation(s) in the native *ileS* gene (Antonio et al., 2002). Furthermore, low-level mupirocin resistance has also been associated with *ileS2* located in the chromosome (Ramsey et al., 1996).

Comprehensive *S. aureus* decolonisation regimes typically involve daily bathing using 4% w/v chlorhexidine body wash, in addition to nasal mupirocin application (Poovelikunnel et al., 2015). Chlorhexidine is a biguanide antiseptic with broad-spectrum antimicrobial activity (Mcdonnell and Russell, 1999). At low concentrations, chlorhexidine is bacteriostatic, disrupting the cell wall and causing leakage of the intracellular content (Mcdonnell and Russell, 1999). At high concentrations, however, chlorhexidine causes coagulation of the cytosol and subsequent bacterial cell death (Mcdonnell and Russell, 1999). In addition to decolonisation, chlorhexidine is used as a skin antiseptic, an oral rinse and a component of some antimicrobial-impregnated catheters (Mcdonnell and Russell, 1999; Poovelikunnel et al., 2015).

Resistance to chlorhexidine is conferred by the Major Facilitator Superfamily (MFS) gene, *qacA,* which encodes a proton gradient-dependent efflux pump and is regulated by *qacR* (Wassenaar et al., 2015). The *qacA* gene was first detected in *S. aureus* in the 1980s and is typically associated with the pSK41/pGO1 plasmid family, although it can be located on the chromosome or other plasmid types (Lyon and Skurray, 1987; Wassenaar et al., 2015). In addition to chlorhexidine, *qacA* encodes resistance to quaternary ammonium compounds (QACs), intercalating dyes acriflavine and ethidium bromide, and diamidines such as propamidine isethionate (Lyon and Skurray, 1987). The *qacB* gene is another MSF member which differs from *qacA* at only seven nucleotide positions and encodes resistance to intercalating dyes and QACs but not to diamidines or chlorhexidine (Horner et al., 2012; Lyon and Skurray, 1987). Accordingly, these genes are often referred to in the literature as *qacA*/*B*. Previous studies have detected *qacA*/*B* in major MRSA lineages such as CC5 and CC8 (Monecke et al., 2011) and global prevalence rates in MRSA range from 1% to 83% (Mayer et al., 2001; McDanel et al., 2013; Noguchi et al., 2005; Shamsudin et al., 2012). Furthermore, the Small Multidrug Resistance (SMR) family gene, *smr* (also known as

qacC), has been associated with low-level chlorhexidine resistance (Kampf, 2016; Wassenaar et al., 2015). In contrast to mupirocin and similar to other antiseptics, standardised guidelines for chlorhexidine susceptibility testing have not been established. This is due in part to the wide range of antiseptic applications and target organisms. Moreover, testing conditions such as time of exposure, amount of organic material present and microbial growth phase have been shown to significantly influence antiseptic susceptibility results, making inter-study comparisons difficult (Guarnier et al., 2010).

Neither *ileS2* or *qacA* are typically reported in CC1 MRSA. Well-defined CC1-MRSA clones include PVL-positive ST1-MRSA-IV (also known as USA400), PVL-negative ST1-MRSA-IV (WA MRSA-1), ST573-MRSA-V (WA MRSA-10) and ST722-MRSA-V (the Bengal Bay Clone or WA MRSA-60) (Monecke et al., 2011). WA MRSA-1 was the first of these four clones to be described and constitutes the oldest known CA-MRSA clone. First recovered in Australia in the late 1980s (Udo et al., 1993), WA MRSA-1 has since been detected in the UAE, Egypt and Europe (Monecke et al., 2011). As suggested by its origin in the community, WA MRSA-1 is not typically associated with multidrug resistance and usually harbours the resistance genes *blaZ* and *erm*(C) (Coombs et al., 2011b). The fusidic acid resistance gene, *fusC* (which is carried on SCC*fus*), is also common among WA MRSA-1 (Coombs et al., 2011b). More recently, WA MRSA-45 and WA MRSA-57 have been described as SLV sub-clones of WA MRSA-1 (Coombs et al., 2011). Specifically, WA MRSA-45 was first isolated in 2006, has been defined as ST872-MRSA-IV/SCC*fus* and is associated with *dfrS1* and *ileS2*, in addition to the resistance genes typically detected in WA MRSA-1 (Coombs et al., 2011b). Similarly, WA MRSA-57 was first isolated in 2007, has been defined as ST1005-MRSA-IV and is associated with the presence of *dfrS1* and *aacA-aphD*, and the absence of *erm*(C), compared to WA MRSA-1 (Coombs et al., 2011b). In the literature however, the presence/absence of SCC*fus* is generally afforded more importance than other potential genotypic variances and thus, SCC*fus*-positive and SCC*fus*-negative PVL-negative CC1-MRSA-IV are often referred to as WA MRSA-1/45 and WA MRSA-1/57, respectively. Furthermore, although an Australian study reported that WA MRSA-45 is associated with *ileS2*, no exemplary isolates have been described and other reports of *ileS2*-encoding WA MRSA-45 are lacking (Coombs et al., 2011b). Both WA MRSA-1 and its SLV sub-clones are typically associated with *spa* type t127 (Coombs et al., 2011b).

While the pandemic ST22-MRSA-IV clone has predominated in Ireland since 2002, CC1 MRSA has not traditionally been associated with Ireland (Kinnevey et al., 2014; Shore et al., 2014). Over the past decade, however, PVL-negative ST1-MRSA-IV has been the most frequently identified CC1-MRSA clone at the NMRSARL, and the prevalence of this clone increased from 0.36% in 2007 to 7.08% in 2015 (personal communication, Dr. Gráinne Brennan, NMRSARL). The latter figure, however, falls to 4.49% if a recent hospital outbreak of high-level mupirocin-resistant (MupR) PVL-negative CC1-MRSA-IV, is discounted (personal communication, Dr. Gráinne Brennan). This protracted outbreak occurred in a large acute-care hospital in Dublin (H1), between 2013 and 2016. During the three years preceding this outbreak (i.e. 2010-2012), the prevalence of high-level mupirocin resistance among BSI MRSA isolates in Ireland averaged 1.99% (personal communication, Dr. Gráinne Brennan). Between 2013 and 2017, however, this figure doubled to 4.0% (personal communication, Gráinne Brennan). The work described in this chapter examined the MupR isolates from this outbreak with three main objectives. Firstly, this work aimed to analyse the *ileS2*-encoding plasmid conferring mupirocin resistance in the outbreak isolates. Secondly, the relatedness of the MupR isolates identified in H1 was investigated in order to confirm/dispute the occurrence of a single clonal outbreak. Finally, in order to determine whether the potential outbreak strain spread beyond H1, the relatedness of the H1 MupR isolates to other PVL-negative CC1-MRSA-IV identified in Ireland during the study period (i.e. between 2013 and 2016) was investigated.

3.2 Materials and Methods

3.2.1 MRSA isolates

All 89 PVL-negative CC1-MRSA-IV isolates identified at the NMRSARL between 2013 and 2016 were investigated (referred to as study isolates). This included 41 MupR patient isolates implicated in the protracted H1 outbreak. Hospital staff used high-level mupirocin resistance as a marker to identify outbreak isolates. All 89 isolates were identified as PVL-negative CC1-MRSA-IV based firstly on their exhibiting *spa* types corresponding to CC1 and secondly, on their harbouring SCC*mec* type IV and lacking *lukS*/*F*-PV, as determined by DNA microarray profiling (see section 3.2.7 below). Two sets of 13 single colonies (referred to as swab isolates) were recovered from the clinical swabs from two separate patients from which study isolates M13/0653 and M15/0221 were also recovered. These swab isolates were used to determine strain variation *in vivo*. All isolates were stored as described in Chapter 2, section 2.2.1.

3.2.2 PCR and gel electrophoresis

PCRs were performed to identify *S. aureus* as MRSA (section 3.2.3) and to confirm the genetic organisation of the *ileS2*-encoding plasmid (section 3.2.10.1). All PCRs were carried out using a Kyratec Thermocycler model SC200 (Kyratec). If the expected product size was over ~4 kb, reaction mixtures were prepared using the Expand Long Template PCR system (Roche Products Ireland Ltd, Dublin, Ireland), according to the manufacturer's instructions. The remaining PCRs were prepared using Go Taq DNA polymerase (Promega, Wisconsin, USA), according to the manufacturer's instructions. Deoxynucleotide triphosphates (dNTPs) and 5x Green GoTaq Flexi Buffer were purchased from the Promega Corporation. DNA Loading Dye and Molecular Weight Markers (100 bp and 1 kb) were purchased from Bioline (Singapore City, Singapore). Oligonucleotides were custom synthesised by Merck and stored as stock solutions of 10 mM at -20°C. GelRed (Biotium, California, USA) was used to fluorescently stain DNA during agarose gel electrophoresis. Agarose gels of 1% (w/v) were prepared by dissolving agarose powder (Merck) in TBE buffer (see Chapter 2, section 2.2.2). Gel electrophoresis was performed for 1 h using a Consort power pack model EV222 (B-2300 Turnhout, Belgium) set at 100 V and 80 mA, a Galileo bioscience gel box (Cambridge, MA, USA) and a well-spacing comb (Thermo Scientific, Waltham, MA, USA). Gels were visualised using ultraviolet light in an Alpha Innotech transilluminator model AVT26U (Protein Simpler, CA, USA) and the AlphaImager mini software (Protein Simple).

3.2.3 Identification of isolates as MRSA

All study isolates were identified as *S. aureus* and MRSA as described in Chapter 2, sections 2.3.2 and 2.3.3, respectively. The swab isolates were recovered from clinical swabs that were processed using Sa*Select* chromogenic agar plates (BioRad) and the Pastorex Staph-Plus kit (Bio-Rad), as described in Chapter 2, section 2.3.1. These isolates were then confirmed as MRSA using a PCR to detect the presence of *mecA*. Reaction volumes of 20 µl were prepared including primers mecA_F (5' – TCCAGATTAACAACTTCACCAGG – 3') and mecA_R (5' – CCACTTCATATCTTGTAACG – 3') (Oliveira and de Lencastre, 2002), 1.5 mM $MgCl_2$, 5x Green GoTaq Flexi buffer and 2.5 U of GoTaq DNA polymerase. The following thermal cycling conditions were used: initial denaturation at 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s, and final extension at 72ºC for 5 min.

3.2.4 Phenotypic susceptibility testing

All study isolates underwent phenotypic susceptibility testing to 20 antimicrobial agents by disk diffusion, as described in Chapter 2, section 2.3.4. To compare phenotypic susceptibility profiles between isolate groups, the two-tailed Fisher's exact test was performed using the GraphPad QuickCalcs website: https://www.graphpad.com/quickcalcs/contingency1/ (accessed October 2018). Results were considered statistically significant if $p < 0.05$. Mupirocin MICs were determined for all MupR study isolates using mupirocin E-test strips (bioMérieux, Nuertlingen, Germany), according to the manufacturer's instructions. The MIC for each isolate was determined as the nearest two-fold dilution above which no visible growth was observed. Isolates exhibiting mupirocin MICs of < 2 mg/L, 2-128 mg/L or > 256 mg/L, were deemed mupirocin-susceptible (MupS), low-level MupR or high-level MupR, respectively (EUCAST, 2018).

3.2.5 *spa* typing

All study isolates underwent *spa* typing as described in Chapter 2, section 2.4.3.

3.2.6 Plasmid analysis

3.2.6.1 Filter mating

Conjugative transfer of the plasmid-encoded *ileS2* gene was performed by filter mating, using the plasmid-free novobiocin-resistant *S. aureus* strain, XU21 (recipient) (Udo and

Jacob, 1998) and the MupR H1 isolate, M14/0355 (donor), as previously described (Woodford et al., 1998). The recipient and donor strains were cultured onto separate Brain Heart Infusion agar (BHIA) plates, as described in Chapter 2, section 2.2.1. Separate Brain Heart Infusion broth (BHIB) volumes of 5 ml were inoculated with a single colony of each resulting culture growth and incubated overnight at 37°C and 200 rpm. Separate BHIB volumes of 5 ml were inoculated with 50 µl of each overnight culture and incubated for 2 h at 37°C and 200 rpm, to allow the cells to reach the mid-logarithmic growth phase. A 50 µl-volume of each culture was mixed together gently in a sterile 1.5 ml tube (Eppendorf). This 100 µl suspension was applied dropwise onto a sterile filter (0.45 µm pore size; Merck Millipore Ltd., Co. Cork, Ireland) which had been placed on a BHIA plate. The BHIA plate was then incubated overnight at 37°C in a static incubator. The filter was transferred into a sterile 50 ml Falcon tube (Corning Life Sciences, New York, USA) containing 1 ml of BHIB, and very gently agitated to remove the culture lawn from the filter. The filter was removed, and the 50 ml-tube was centrifuged at 2,000 x *g* for 5 min. The supernatant was removed, and the pellet was resuspended in 500 µl of BHIB. The 50-ml tube was then incubated for 2 h at 37°C and 200 rpm. The resuspended bacterial cells were plated onto both BHIA and BHIA supplemented with mupirocin (100 mg/L; GlaxoSmithKline, Dublin, Ireland) and novobiocin (10 mg/L; Merck). These agar plates were then incubated for 48 h at 37°C. Putative transconjugant derivatives of XU21 were identified as the colonies that grew on the selective agar. DNA microarray profiling was used to confirm the presence of *ileS2* in three separate colonies recovered on selective medium (see section 3.2.7 below).

3.2.6.2 Plasmid Curing

Isolate M14/0355 was cultured onto BHIA, as described in Chapter 2, section 2.2.1. A 5 ml-volume of BHIB was inoculated with a single colony of the resulting growth and incubated overnight at 37°C. A fresh 5 ml volume of BHIB was inoculated with 100 µl of a 10^{-4} dilution of the overnight broth culture and incubated overnight at 43°C and 200 rpm for 24 h, for four consecutive rounds. The final overnight culture underwent serial dilution to a concentration of 10^{-8}. The 10^{-4} to 10^{-8} dilutions were plated onto BHIA and incubated overnight at 37°C. The BHIA plates (i.e. the master plates) were then refrigerated at approximately 4°C for 2-3 h, to allow the colonies to become firmer for replica plating (Fig. 3.1). A heavy-duty circular plastic block (diameter 7.6 cm), with a sterile piece of velvet material (approximately 15 cm x 15 cm) secured in place by a metal ring was used as a replica plating device. A master plate with approximately 50-100 colonies was placed

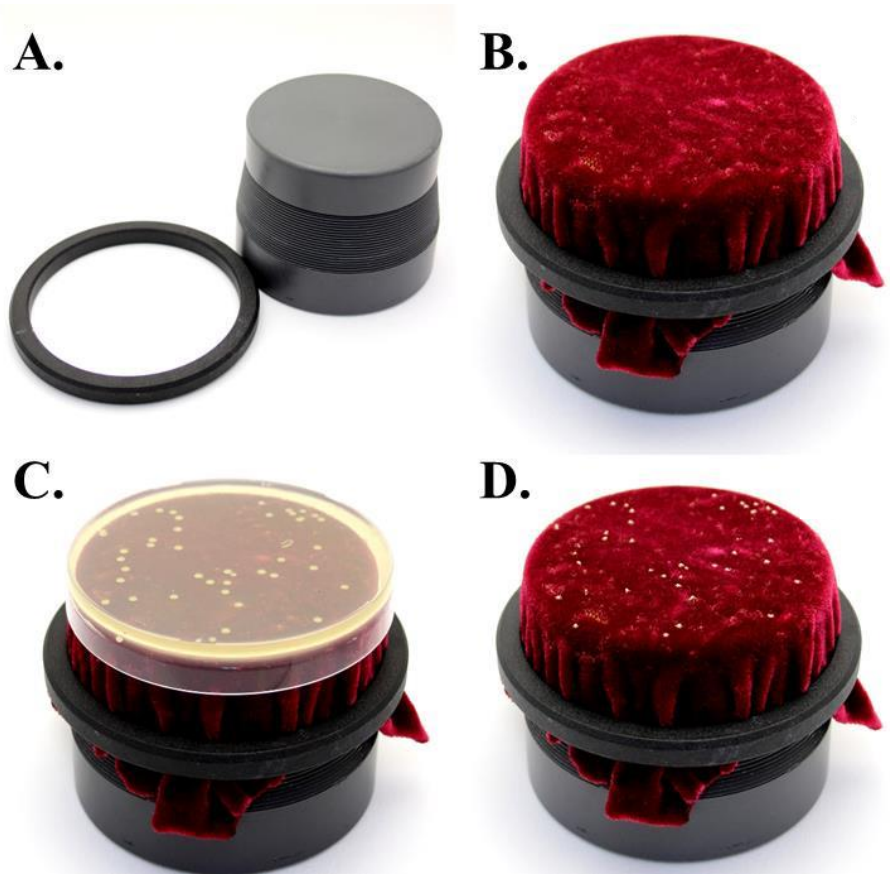


Figure 3.1 A photograph showing the replica plating process used during plasmid curing. **A.** The replica plating device consisted of a circular block of heavy duty plastic with a diameter of 7.6 cm and a metal ring. **B.** A sterile piece of velvet material (15 cm x 15 cm) was secured on the replica plating device using the metal ring. **C.** A Brain Heart Infusion agar (BHIA) plate with 50-100 colonies was placed face down onto the sterile velvet. **D.** The bacterial colonies were imprinted onto the sterile velvet. A fresh BHIA plate supplemented with 100 mg/L mupirocin was placed onto the velvet and gentle force was applied to the back of the plate to allow transfer of the bacterial growth. The colonies from which the *ileS2*-encoding plasmid had been lost were identified by their inability to grow on the selective media. Adapted from Brennan, 2013.

face down onto the replica plating device and gentle pressure was applied to the bottom of the plate to transfer part of the colony growth onto the velvet. The master plate was then removed and a fresh BHIA plate supplemented with mupirocin (100 mg/L) was placed onto the imprinted velvet with gentle pressure, to allow transfer of the bacterial growth onto the agar plate. The process was repeated with a regular BHIA plate. Master plates were stored at 4ºC and replica plated plates were incubated for 18 h at 37ºC. Putative cured derivatives of M14/0355, identified as the colonies that grew on the non-selective BHIA plate but not the selective BHIA plate, were then purified by subculturing. DNA microarray profiling was used to confirm the absence of *ileS2* in one cured derivative (see section 3.2.7 below).

3.2.7 DNA microarray profiling

All 89 study isolates, three putative transconjugants and one cured derivative underwent DNA microarray profiling using the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH]), according to the manufacturer's instructions. All buffer, primers, reagents and microarray chips required to perform the protocol are included in the kit. The microarray chips are mounted in 8-well microtitre strips, and each chip contains 336 probes which correspond to the allelic variants of 185 different genes. These genes include the species marker, virulence-associated, antimicrobial resistance and SCC-associated marker genes that ultimately determine the DNA microarray profile of an isolate. Detailed descriptions of the relevant genes, primers and probes have been previously described (Monecke et al., 2008).

3.2.7.1 Linear PCR amplification and biotin-dUTP labelling

The target sequences present in each DNA sample were amplified and labelled with biotin-16-dUTP. Only one reverse primer was included per target sequence resulting in the generation and labelling of single stranded DNA products. A master mix was prepared by combining 4.9 µl of B1 labelling buffer and 0.1 µl of B2 enzyme reagent per sample. A 5 µl aliquot of the master mix was combined with 5 µl of template DNA. Amplification was performed using a G-storm GSI Thermocycler (G-Storm) and the following thermal cycling conditions: initial denaturation at 96ºC for 5 min, followed by 45 cycles of 96ºC for 2 s, 50ºC for 20 s and final extension at 72ºC for 30 s.

3.2.7.2 Hybridisation of PCR products to array probes

Each microtitre well was washed with 200 µl of ultrapure water. Following this, 100 µl of C1 hybridisation buffer was added to each well and the microarray strip was incubated for 2 min at 55ºC, with shaking at 550 rpm. All non-room temperature incubation steps were carried out in a BioShake iQ thermoshaker (Q instruments, Jena, Germany). Individual hybridisation mixtures were prepared containing 10 µl of each PCR product and 90 µl of C1 hybridisation buffer. These mixtures were distributed into individual microarray wells and the microarray strip was incubated for 60 min at 55ºC and 550 rpm to allow hybridisation to occur.

3.2.7.3 Horseradish-peroxidase (HRP)-conjugation and staining

Following hybridisation, the microtitre wells were washed three times with 200 µl C2 wash buffer. A mixture containing C3 streptavidin-HRP reagent and C4 conjugate buffer at a ratio of 1:100 was prepared. A 100 µl volume of this C3/C4 mixture was added to each microtitre well and the microarray strip was incubated for 10 min at 30ºC and 550 rpm, to enable binding of the streptavidin-HRP to the biotin-16-dUTP target. Each microtitre well was then washed with 200 µl of C5 wash buffer. To stain the biotin-HRP conjugate, 100 µl of D1 tetramethylbenzidine HRP substrate was added to each microtitre well and the microarray strip was incubated at room temperature for 5 min without agitation. The D1solution was fully removed from each well prior to chip analysis.

3.2.7.4 Data analysis

An image of each microarray chip was recorded using an ArrayMate reader (Abbott [Alere Technologies GmbH]) and the IconoClust software package (Abbott [Alere Technologies GmbH]) was used to interpret the raw data generated. For each microarray, the quality of these data was deemed sufficient if the microarray chip was intact and the staining control was positive. Raw data were interpreted as positive, negative or ambiguous using a previously described algorithm (Monecke et al., 2008). Specifically, signal strength breakpoints were defined using the average signal strength of the control spots and species markers. Signals were then deemed positive if >33%, ambiguous if between 25-33% and negative if <25%, of this average. Iconoclast (Abbott [Alere Technologies GmbH]) was used to compare the microarray profiles of the query isolates to those of the previously characterised reference *S. aureus* strains in the ArrayMate database. This comparison allowed each isolate to be assigned a MLST CC and SCC*mec* type. The Results Collector (Abbott [Alere Technologies GmbH]) tool was used to combine the ArrayMate data into

two CSV files which were imported into Microsoft Excel v15.4 for further processing and analysis.

3.2.8 WGS

All study and swab isolates underwent WGS as described in Chapter 2, section 2.5 (Table 3.2).

3.2.9 Whole genome analysis

3.2.9.1 de novo assembly

Sequence read sets were assembled into contigs using the Velvet genome assembler v1.2.10, incorporated into BioNumerics v7.6 (Applied Maths).

3.2.9.2 MLST

Isolate contig sets underwent conventional MLST using Ridom SeqSphere+ version 4.1 (Ridom GmbH), as described in Chapter 2, section 2.6.3.

3.2.9.3 wgMLST

Isolate genomes underwent wgMLST using BioNumerics v7.6 (Applied Maths), as described in Chapter 2, section 2.6.8. Distance matrices based on cgMLST loci were generated for each of the swab isolate sets.

3.2.9.4 MST generation

Two MSTs were constructed, based on cgMLST loci, as described in Chapter 2, section 2.6.10. The first MST generated included all 89 study isolates investigated. The second MST generated included a selection of 78 study isolates, chosen based on their close relatedness, as determined using the first MST.

3.2.9.5 SNV analysis

All 78 isolates included in the second MST underwent SNV analysis using a study-specific reference sequence. Isolate M15/0029 was chosen as the reference due to its central position in the MST cluster. SNV analysis was performed as described in Chapter 2, section 2.6.7. SNVs were called exclusively in positions shared by all samples. Only SNVs with at least 5x coverage, including 1x coverage in each direction, were considered. Potentially indel-related SNVs, occurring within 12 bp of each other, were removed.

Table 3.2 Whole-genome sequencing quality assurance data

Quality parameter	Average result
Trimmed read quality	36.0
N50[a]	40293.2
No. of contigs per isolate	192.3
Assembly coverage	139.7
% core-genome loci present[b]	98.4

[a]The minimum contig length required to cover half the genome.

[b]According to the previously defined core-genome multilocus sequence typing scheme (Leopold et al., 2014).

Positions with ambiguous base calls were excluded.

3.2.10 *ileS2* plasmid sequence analysis

3.2.10.1 Confirmation of genetic organisation

Isolate M14/0355 previously underwent SMRT sequencing as part of a different study in this laboratory (Lazaris, 2016). This resulted in the generation of the entire *ileS2*-encoding plasmid on a single contig. In order to ensure that the SMRT sequence did not contain any false insertions or deletions, eight overlapping primer sets (Table 3.3) were designed across the entire SMRT sequence and PCRs were performed with the intention of generating amplimers of known sizes. For the reaction involving primers 0F and 0R, a 20 µl reaction volume was prepared including 1.5 mM $MgCl_2$, 5x Green GoTaq Flexi buffer and 2.5 U of GoTaq DNA polymerase. The following thermal cycling conditions were used: initial denaturation at 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s, and final extension at 72ºC for 5 min. For the reactions involving primers 1-8F and 1-8R, a 50 µl reaction volume was prepared including Expand Long Template buffer 3 and Expand Long Template enzyme mix. The following thermal cycling conditions were used: initial denaturation at 92ºC for 2 min, followed by 10 cycles of 92ºC for 10 s, 59ºC for 30 s, 68ºC for 4 min, followed by 20 cycles of 92ºC for 15 s, 59ºC for 30 s, 68ºC for 5.5 min and final extension at 68ºC for 7 min. As primers 0F and 0R were designed to confirm the plasmid "ends" (and all other amplimers were of the expected size), the corresponding PCR product was purified using the GenElute PCR Clean-Up kit (Merck) and sequenced commercially (Source Bioscience, Waterford, Ireland) using the Sanger method (Sanger et al., 1997). The resulting DNA sequence was analysed using the ApE plasmid editor v2.0.45 (http://jorgensen.biology.utah.edu/wayned/ape/).

3.2.10.2 Annotation and map construction

All ORFs comprising at least 30 codons were identified using ORF Finder (http://www.bioinformatics.org/sms2/orf_find.html). If possible, ORFs were annotated using the publicly available National Centre for Biotechnology Information (NCBI) BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A genetic map of the plasmid was constructed using SnapGene v4.1.9 (https://www.snapgene.com).

Table 3.3 Oligonucleotide primers used to confirm the genetic organisation of plasmid p140355

Primer name	Sequence (5'-3')	Nucleotide position[a]	Region amplified	Amplimer size (bp)
0F	GTTGAGGTGGTTGAATGGATTG	41986-42499	*traL* subsection	513
0R	CTGCCATAGTCCCCATAGAACC			
1F	GGGGACTATGGCAGGTAG	42485-2549	mid-*traL* – intergenic region between ORF6 and ORF7	5988
1R	CATTATCAGTTTGACGTGTTATTAG			
2F	GATAATGAAAATAGAAGAACTGTGC	2543-8559	intergenic region between ORF6 and ORF7 – mid-*ileS2*	6016
2R	AACCTCTAATTCAACTGGTAAGC			
3F	TGGCTTACCAGTTGAATTAGAG	8535-14556	mid-*ileS2* - intergenic region between *parB* and ORF20	6021
3R	AGCTCATATTTGTGTTCTCAGATC			
4F	AGGATCTGAGAACACAAATATGAG	14351-20546	intergenic region between *parB* and ORF20 – mid-ORF26	6015
4R	CATCTTGTTGTGTAAAATCGTC			
5F	ACAGTGATATTAATGAAGTTGACG	20505-26505	mid-ORF26 – mid-*nes*	6000
5R	AATAGTAACAACAATACCTAAAGCG			
6F	TCGCTTTAGGTATTGTTGTTAC	26480-32378	mid-*nes* – mid-*traD*	5898
6R	TGTCTTAATCATTGGCTCATC			
7F	TGGAGGTTTAAATATTACTGGTG	32255-38249	mid-traD – mid-*traI*	5994
7R	AATGACGTGATAAGTTAATTCCTAC			
8F	AGGGCTAGAGAATTAAGTGATTG	38198-42001	mid-*traI* – mid-*traL*	3803
8R	ATTCAACCACCTCAACTTTC			

[a]Based on GenBank entry KY465818.

3.2.10.3 Sequence comparisons and GenBank upload

The read sets of the remaining *ileS2*-encoding isolates were mapped against the SMRT sequence using the Burrows-Wheeler aligner (http://arxiv.org/abs/1303.3997). Artemis sequence viewer (https://www.sanger.ac.uk/science/tools/artemis) was used to visually assess the mapping of reads. The NCBI BLAST database was used to compare the SMRT sequence to other publicly available sequences. The SMRT sequence (p140355) was submitted to GenBank (accession number: KY465818).

3.3 Results

3.3.1 Description of MRSA isolates investigated

All isolates were characterised as ST1-MRSA-IV, the vast majority (87/89) of which were identified as *spa* type t127 (*spa* repeat succession: 07-23-21-16-34-33-13), while those remaining were identified as t922 (07-23-21-16-33-13). All epidemiological information associated with the 89 study isolates investigated is detailed in Table 3.4. These 89 isolates were recovered in 17 different hospitals (H1-H17), three different healthcare facilities (HCF-1, HCF-2 and HCF-3) and the community. Isolates recovered in the community were from patients attending general medical practitioners (GPs; n = 9), outpatient departments (n = 1) or accident and emergency departments (n = 1). In addition to the 41 MupR patient isolates implicated in the H1 outbreak, the 89 study isolates included five MupS isolates from H1. The majority (44/89) of isolates were recovered from colonisation sites (nose, throat and/or groin), 12/89 were recovered from SSTIs, 9/89 were recovered from invasive infections, 23/89 were recovered from unknown colonisation/infection sites, and 1/89 was recovered from the environment. One isolate per patient was included with the exception of three instances in which an isolate was recovered from a single patient at two different time-points (i.e. there were three "isolate pairs"). The average patient age was 61.7 years (range: 2 days – 96 years).

3.3.2 One large cluster (including two sub-clusters) and eleven outliers identified

The vast majority (78/89) of isolates grouped into one major cluster in a cgMLST-based MST, while 11 outlier isolates dispersed throughout the remainder of the tree (Fig. 3.2). The cluster isolates exhibited a maximum of 182 pairwise allelic differences and were differentiated from the outliers by a minimum allelic distance of 217. Two sub-clusters (I and II), which were differentiated by an allelic distance of 58, were identified within the major MST (Fig. 3.3). Sub-cluster I included 57 isolates which exhibited an average of 25 pairwise allelic differences (min, 1; max, 98). Sub-cluster II included 19 isolates which exhibited an average of 61 pairwise allelic differences (min, 2; max, 82). Sub-clusters were defined as isolates groups within the major MST cluster in which no isolate was differentiated from the cluster by an allelic distance ≥ 58 and hence, two "intra-cluster outliers" were identified (Fig 3.3). These intra-cluster outliers, M14/0597 and M16/0223, differed from the remaining sub-cluster II isolates by allelic distances of 65 and 62, respectively. The two sets of swab isolates exhibited internal pairwise allelic distances of 0-4 and 0-17.

Table 3.4 Epidemiological data associated with the 89 PVL-negative CC1-MRSA-IV isolates investigated recovered in Ireland between 2013 and 2016

Isolate[a]	Date of recovery	Source[b]	Presentation	MST position[c]
M13/0404	13/06/2013	GP	SSTI	Sub-cluster II
M13/0653	05/11/2013	H1_A	Colonisation	Sub-cluster I
M13/0671	15/11/2013	H1_A	Colonisation	Sub-cluster I
M14/0015	24/10/2013	H16_A	BSI	Outlier
M14/0046	11/05/2014	GP	Colonisation	Sub-cluster I
M14/0103	16/02/2014	H1_D	Osteomyelitis	Sub-cluster I
M14/0125	26/02/2014	H1_A	Colonisation	Sub-cluster I
M14/0279	19/05/2014	H1_A	SSTI	Sub-cluster I
M14/0355	30/05/2014	H1_A	SSTI	Sub-cluster I
M14/0373	11/05/2014	H1_A	Colonisation	Sub-cluster I
M14/0425	24/06/2014	H1_A	Colonisation	Sub-cluster I
M14/0466	02/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0467	10/05/2014	H2_B	Unknown	Sub-cluster II
M14/0480	01/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0481	01/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0586	08/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0597[d]	14/07/2014	H6_X	Unknown	Intra-cluster outlier
M14/0602	16/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0603	16/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0648	25/07/2014	GP	SSTI	Outlier
M14/0656	28/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0660	28/07/2014	H1_A	Colonisation	Sub-cluster I
M14/0664	05/08/2014	H1_A	Colonisation	Sub-cluster I
M14/0681	15/08/2014	H1_A	SSTI	Sub-cluster I
M14/0695	21/08/2014	H1_A	Colonisation	Sub-cluster I
M14/0697	20/08/2014	H2_B	Unknown	Sub-cluster II
M14/0713[e]	Unknown	H1_K	N/A	Sub-cluster I
M14/0845	23/09/2014	H5_A	Colonisation	Sub-cluster II
M14/0857	23/09/2014	H5_A	Colonisation	Sub-cluster II
M14/0868	16/10/2014	H1_A	Colonisation	Sub-cluster I
M14/0876	22/10/2014	H1_A	Colonisation	Sub-cluster I
M14/0877	27/10/2014	H1_A	Colonisation	Sub-cluster I
M14/0878	23/10/2014	H1_A	Colonisation	Sub-cluster I
M14/0886	29/10/2014	H7_B	Unknown	Outlier
M14/0892	22/10/2014	H1_A	Colonisation	Sub-cluster I
M14/0953	27/11/2014	H14_A	BSI	Outlier

Table 3.4 continued overleaf

Isolate[a]	Date of recovery	Source[b]	Presentation	MST position[c]
M14/0965[f]	25/11/2014	H1_A	Graft infection	Sub-cluster I
M14/0966	17/11/2014	H1_A	Colonisation	Sub-cluster I
M14/0967	28/10/2014	H1_D	Colonisation	Sub-cluster I
M14/0968[g]	25/11/2014	H17_A	Colonisation	Sub-cluster I
M14/0992[d]	19/12/2014	H1_A	Colonisation	Sub-cluster I
M14/0993[g]	02/12/2014	H3_A	BSI	Sub-cluster I
M14/0994	17/12/2014	H7_A	Unknown	Outlier
M15/0029	15/11/2015	H1_C	Colonisation	Sub-cluster II
M15/0030	25/02/2014	H1_J	Colonisation	Sub-cluster II
M15/0031[g]	01/05/2014	HCF-1	Colonisation	Sub-cluster II
M15/0067	28/01/2015	H12_A	Colonisation	Outlier
M15/0068	20/01/2015	H1_A	BSI	Sub-cluster I
M15/0127	18/08/2014	H3_D	Unknown	Sub-cluster I
M15/0138	11/02/2015	H1_B	Colonisation	Sub-cluster I
M15/0148[h]	04/01/2015	H1_E	Colonisation	Sub-cluster I
M15/0149	25/01/2015	H1_A	Colonisation	Sub-cluster II
M15/0154	16/02/2015	H1_E	Colonisation	Sub-cluster I
M15/0161	06/02/2015	H10_A	Colonisation	Outlier
M15/0164	13/01/2015	H2_A	Unknown	Sub-cluster II
M15/0201	22/08/2014	H3_B	Unknown	Sub-cluster I
M15/0206	10/03/2015	H1_B	Colonisation	Sub-cluster I
M15/0213[d]	16/03/2015	GP	SSTI	Sub-cluster I
M15/0221	17/03/2015	H1_F	Unknown	Sub-cluster I
M15/0222	24/03/2015	H1_B	Colonisation	Sub-cluster I
M15/0223[f]	24/03/2015	H1_B	Graft infection	Sub-cluster I
M15/0245	01/04/2015	GP	SSTI	Sub-cluster II
M15/0266	01/04/2015	H13_X	Unknown	Sub-cluster I
M15/0286	30/04/2015	H4_A	Unknown	Sub-cluster II
M15/0307	11/05/2015	H3_C	Unknown	Sub-cluster II
M15/0337	27/05/2015	H1_K	SSTI	Sub-cluster I
M15/0371	04/06/2015	OPD	Unknown	Sub-cluster II
M15/0382	17/06/2016	GP	SSTI	Sub-cluster II
M15/0384	17/06/2015	H11_A	Unknown	Sub-cluster II
M15/0429[g]	06/07/2015	HCF-2	Unknown	Sub-cluster I
M15/0443	13/07/2015	H15_A	Unknown	Sub-cluster II
M15/0540[i]	31/07/2015	H1_A	Colonisation	Sub-cluster I
M15/0541[i]	23/08/2015	H1_I	Colonisation	Sub-cluster I
M15/0575	21/09/2015	H4_B	Unknown	Sub-cluster I

Table 3.4 continued overleaf

Isolate[a]	Date of recovery	Source[b]	Presentation	MST position[c]
M15/0609	25/09/2015	HCF-3	Unknown	Sub-cluster I
M15/0614	10/09/2015	H1_C	BSI	Sub-cluster I
M15/0637[g,h]	10/09/2015	GP	Colonisation	Sub-cluster I
M15/0640	14/10/2015	H9_A	Unknown	Outlier
M15/0659	20/10/2015	H4_C	Unknown	Sub-cluster I
M15/0724	04/12/2015	H1_H	Colonisation	Sub-cluster I
M16/0002	24/12/2015	GP	SSTI	Sub-cluster II
M16/0116	05/02/2016	H8_A	Colonisation	Sub-cluster II
M16/0123	08/01/2016	H6_A	Unknown	Outlier
M16/0139	09/02/2016	H3_A	Unknown	Sub-cluster I
M16/0141	06/02/2016	H1_G	Colonisation	Sub-cluster I
M16/0183	02/03/2015	ED	BSI	Outlier
M16/0188	08/03/2016	H2_A	Unknown	Outlier
M16/0219	31/03/2016	GP	SSTI	Sub-cluster I
M16/0223	31/03/2016	H6_X	SSTI	Intra-cluster outlier

[a]The 41 MupR isolates originally implicated in the H1 outbreak are indicated in bold.

[b]For the isolates recovered in hospitals H1-H17, the ward of recovery is indicated by a letter. The letter "X" indicates that the ward of recovery is unknown.

[c]As detailed in the results section of this chapter, the majority of isolates grouped into one major cgMLST-based MST cluster (including two sub-clusters and two intra-cluster outliers), while the remaining isolates were termed "outliers".

[d]Isolates M14/0597 and M14/0992 were recovered from HCWs in H6 and H1, respectively. Isolate M15/0213 was recovered from a H1 HCW who presented to a GP.

[e]Isolate M14/0713 was recovered from the environment in H1.

[f]Isolates M14/0965 and M15/0223 were recovered from the same patient.

[g]Patient had recent hospital H1 admission history.

[h]Isolates M15/0148 and M15/0637 were recovered from the same patient.

[i]Isolates M15/0540 and M15/0541 were recovered from the same patient.

Abbreviations: BSI, bloodstream infection; ED, emergency department; GP, general medical practitioner; HCF, healthcare facility; H, hospital; MST, minimum spanning tree; MupR, mupirocin-resistant; OPD, outpatient department; SSTI, skin and soft tissue infection.

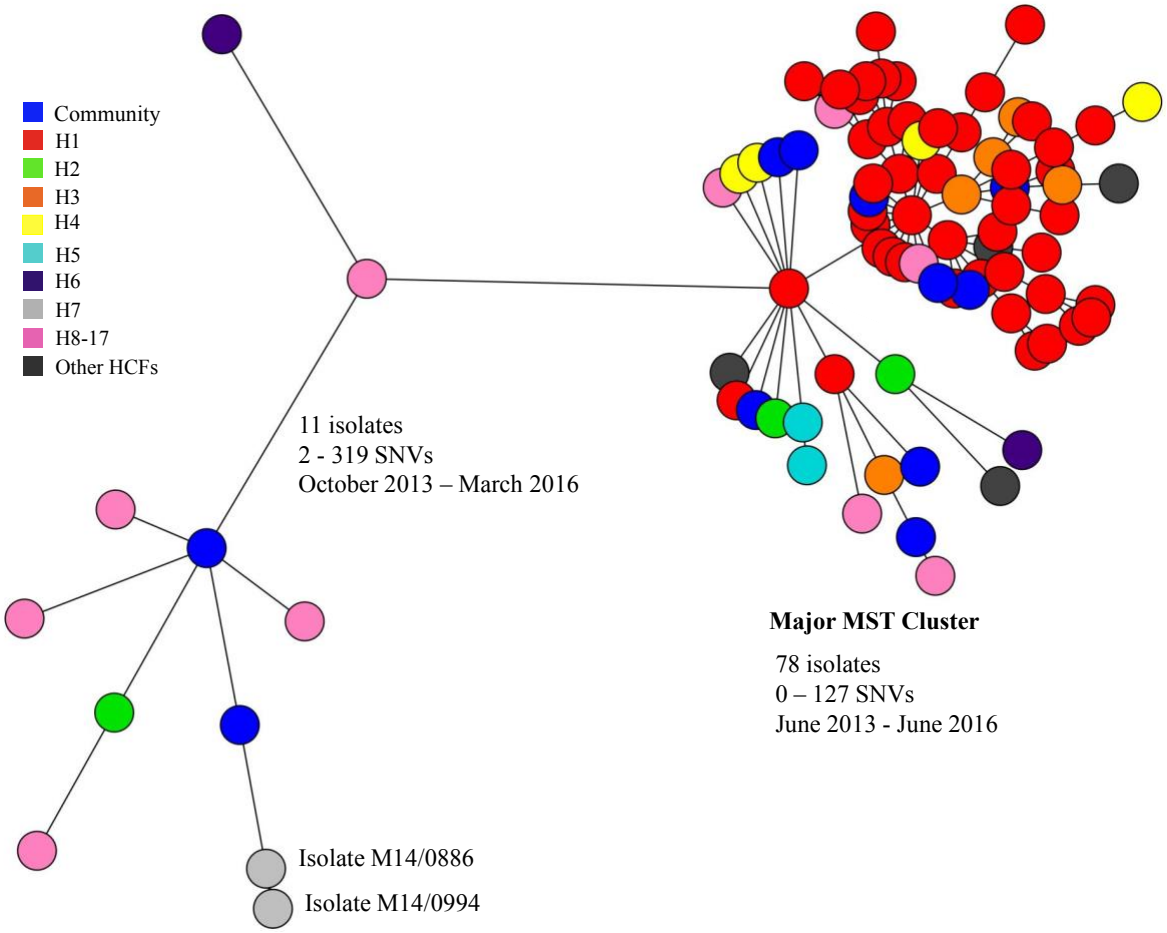


Figure 3.2 A MST based on the cgMLST profiles of 89 CC1-MRSA-IV isolates recovered in Ireland between 2013 and 2016. The locations from which the isolates were recovered are indicated in the colour legend. One isolate was recovered from each of hospitals H8-H17. Abbreviations: cgMLST, core-genome multilocus sequence typing; H, hospital; HCFs, healthcare facilities; MST, minimum spanning tree; SNV, single nucleotide variation.

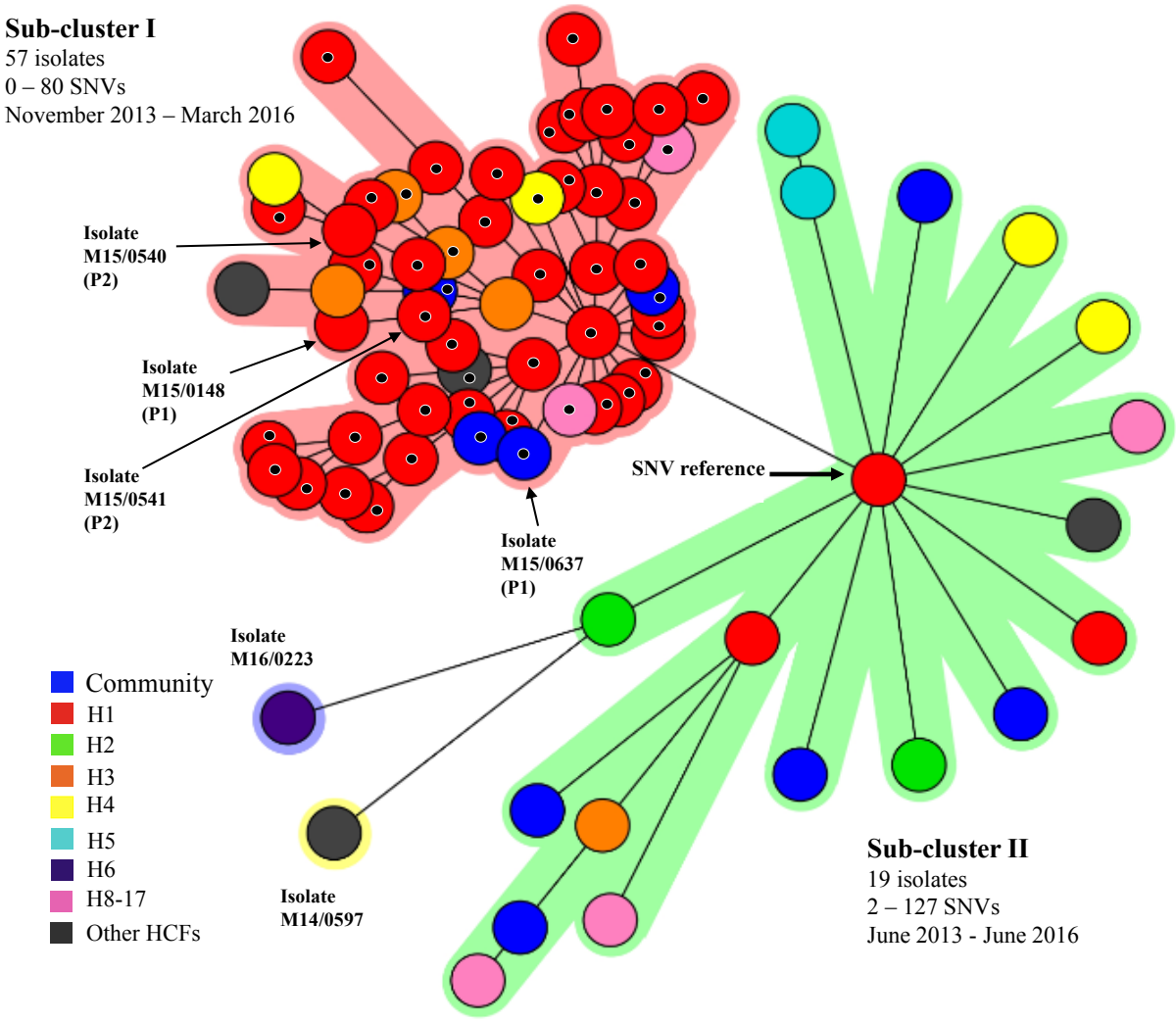


Figure 3.3 A MST based on the cgMLST profiles of the 78 CC1-MRSA-IV isolates in the major MST cluster shown in Figure 3.2. Two sub-clusters were identified within the major cluster: sub-cluster I; highlighted in pink and sub-cluster II; highlighted in green. The two intra-cluster outliers, M16/0223 and M14/0597, are highlighted in purple and yellow, respectively. The isolate pairs recovered from patient P1 (M15/0148; *ileS2*-negative/MupS and M15/0637; *ileS2*-positive/MupR) and patient P2 (M15/0540; *ileS2*-positive/MupS and M15/0541; *ileS2*-positive/MupR) are indicated. The locations from which the isolates were recovered are indicated in the colour legend. One isolate was recovered from each of hospitals H8-H17. The isolates that exhibited high-level mupirocin resistance are indicated by the black dots. Abbreviations: cgMLST, core-genome multilocus sequence typing; H, hospital; HCFs, healthcare facilities; MupS, mupirocin-susceptible; MupR, mupirocin-resistant; SNV, single nucleotide variation.

3.3.3 Sub-cluster I

Sub-cluster I isolates were recovered in three different geographic regions of Ireland over a 28-month period (November 2013 to March 2016; Fig. 3.4). This included the majority (43/46) of H1 isolates (including all 41 MupR H1 isolates), and isolates recovered in H3 (4/5), H4 (2/3), H13 (1/1), H17 (1/1), HCF-2 (1/1), HCF-3 (1/1) and the community (4/11; Fig. 3.3). The vast majority (55/57) of sub-cluster I isolates were recovered from patients, while one isolate each was recovered from a H1 HCW and the H1 environment. Isolate M15/0213 was recovered from a H1 HCW who presented to a GP and was therefore recorded as a community isolate (Table 3.4). The HCF-2 isolate (M15/0429) and community isolate M15/0637 were recovered from patients who had been recently transferred or discharged from H1, respectively (Table 3.4).

All sub-cluster I isolates were identified as MDR, exhibiting phenotypic resistance to aminoglycosides, ß-lactams, MLS_B (macrolides, lincosamides and streptogramin B) compounds, mupirocin and/or tetracycline (Fig. 3.5). These phenotypes were mediated by *aadD*/*aphA3*, *blaZ*, *erm*(C)/*lnuA*/*vga*(A), *ileS2* and *tet*(K), respectively, all of which were detected in sub-cluster I at varying frequencies (Table 3.5). Mupirocin resistance (which was exclusive to sub-cluster I) was detected in the vast majority (50/57) of sub-cluster I isolates, all of which exhibited mupirocin MICs $\geq$ 1024 mg/L. These MupR isolates were recovered largely (40/50) in hospital H1 but also in hospitals H3 (2/50), H4 (1/50), H13 (1/50), H17 (1/50), HCF-2 (1/50) and the community (4/50; Fig. 3.3). The *ileS2* gene was detected in all MupR isolates and in two phenotypically MupS isolates (M15/0201 from H3 and M15/0540 from H1). A single adenine insertion was identified at nucleotide position 283 of the *ileS2* gene in each of these MupS isolates, which resulted in a downstream frameshift mutation and premature stop codon. The *ileS2* gene was not detected in any isolates outside of sub-cluster I. In addition to *ileS2*, resistance genes *aadD*, *fosB*, *fusB*, *lnu*(A) and *vga*(A), were also detected exclusively in sub-cluster I, although generally at low frequencies (Table 3.5). Finally, the *qacA* gene was detected in 53/57 of sub-cluster I isolates, 51 of which also harboured *ileS2*. One isolate harboured *ileS2* but not *qacA*. Based on their genotypic profiles, DNA microarray analysis indicated that all sub-cluster I isolates were affiliated with WA MRSA-1/57.

A novel conjugative *ileS2*-encoding plasmid, designated p140355, was identified in isolate M14/0355. This plasmid was characterised as a 45,924 bp circular plasmid of the

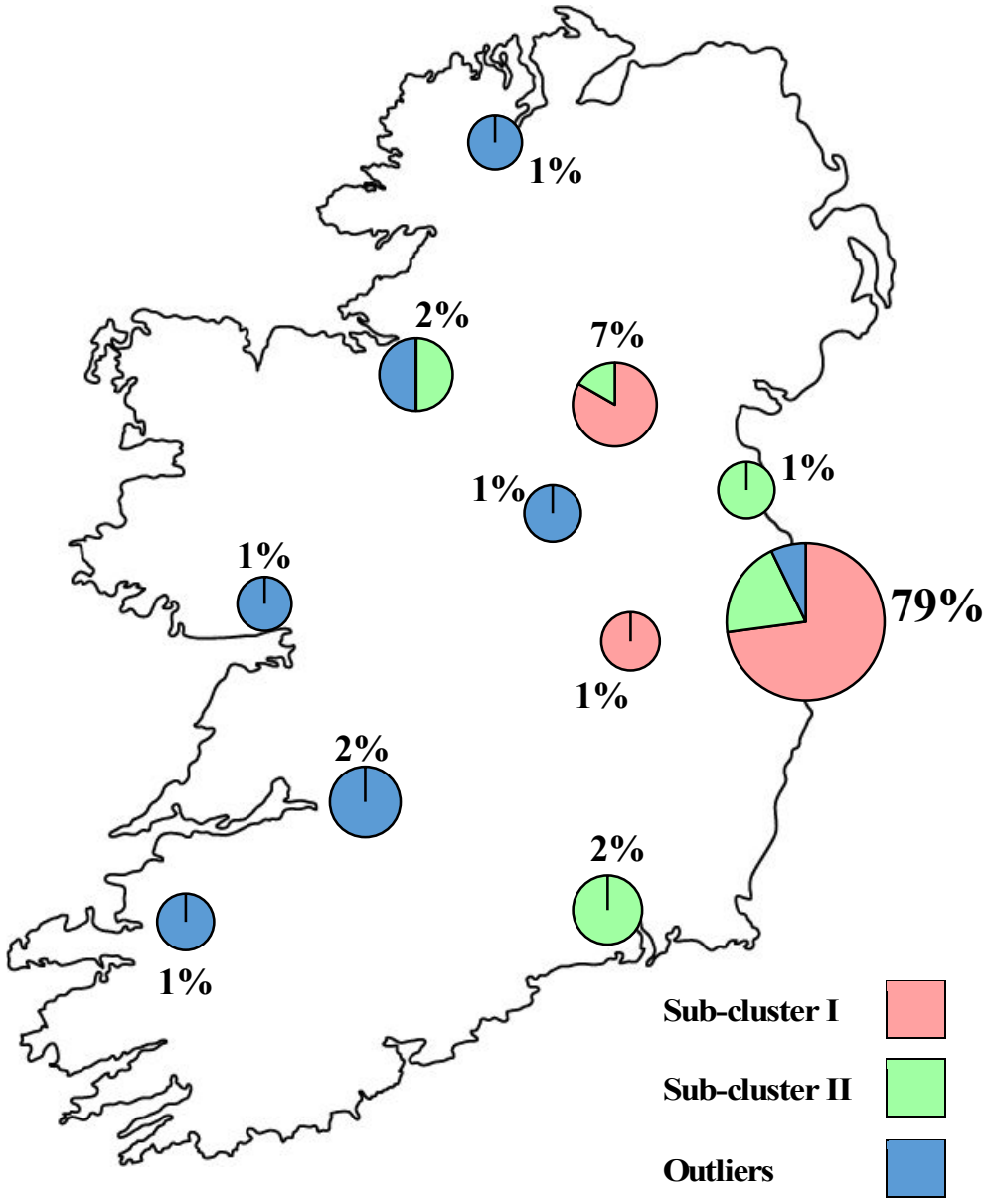


Figure 3.4 The regions of Ireland in which 89 CC1-MRSA-IV isolates were recovered between 2013 and 2016**.** The majority of isolates grouped into one major cluster in a core-genome multilocus sequencing typing-based minimum spanning tree, while the remaining 11 isolates were identified as outliers (Fig. 3.2). The major cluster included two sub-clusters and two intra-cluster outliers. The intra-cluster outliers are included in sub-cluster II in the present figure. Percentages are expressed as a proportion of the total number of isolates investigated. Pie charts are not to scale.

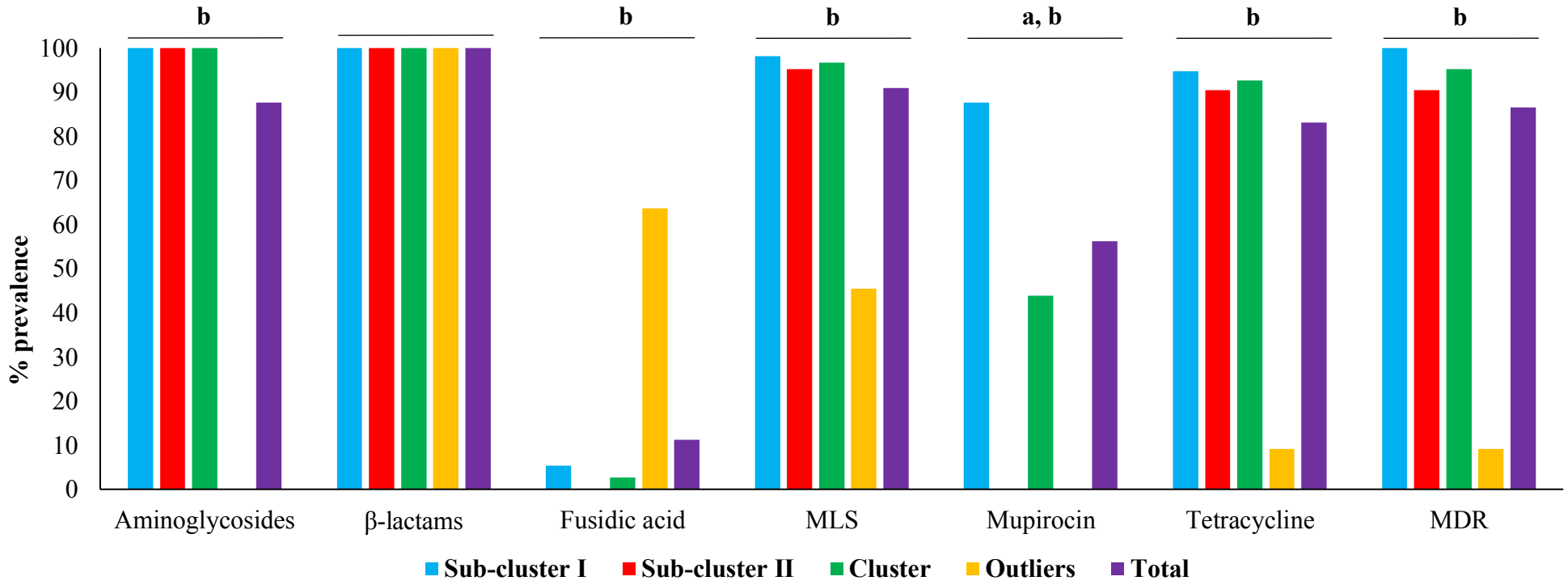


Figure 3.5 The phenotypic resistance patterns of the 89 CC1-MRSA-IV isolates recovered in Ireland between 2013 and 2016. The majority of isolates grouped into one major cluster in a cgMLST-based minimum spanning tree, while the remaining 11 isolates were identified as outliers. The major cluster included two sub-clusters and two intra-cluster outliers. The intra-cluster outliers are included in sub-cluster II in the present figure. The letter "a" indicates statistically significant results between isolates within sub-clusters I and II. The letter "b" indicates statistically significant results between isolates in the cluster (i.e. both sub-clusters) and the outliers. For all statistically significant results, $p < 0.0001$. Abbreviations: MLS, macrolides, lincosamides and streptogramin compounds; MDR, multidrug-resistant.

Table 3.5 The resistance genes detected in the 89 CC1-MRSA-IV isolates recovered in Ireland between 2013 and 2016.

	Percentage prevalence			
Gene	**Sub-cluster I (n = 57)**	**Sub-cluster II (n = 21)**	**Outliers (n = 11)**	**Total (n = 89)**
aadD	2	0	0	1
aphA3	100	100	0	88
blaZ	100	100	82	98
erm(C)	100[a]	100[a]	27	91
fosB	7	0	0	4
fusB	4	0	0	2
fusC	0	0	64	8
lnu(A)	2	0	0	1
ileS2	91[b]	0	0	58
qacA	93	0	0	60
sat	100	100	0	88
tet(K)	98[c]	100[c]	0	87
tet(M)	0	0	9	1

[a]The *erm*(C) sequence was identified in the contig sets of three sub-cluster I and four sub-cluster II isolates identified as *erm*(C)-negative using DNA microarray profiling. All other cluster isolates were identified as *erm*(C)-positive using DNA microarray profiling.

[b]A premature stop codon was identified in the *ileS2* sequence of two isolates (M15/0201 and M15/0540).

[c]The *tet*(K) sequence was identified in the contig sets of 22 sub-cluster I and 15 sub-cluster II isolates identified as *tet*(K)-negative using DNA microarray profiling. All other sub-cluster II isolates and 34/35 of the remaining sub-cluster I isolates were identified as *erm*(C)-positive using DNA microarray profiling.

pSK41/pGO1 family, exhibiting a GC content of 28.6% (Fig. 3.6). The plasmid backbone accounted for approximately 27.5 kb of the p140355 sequence, while the remaining 18.5 kb comprised the accessory region (Fig. 3.6). A total of 54 ORFs were identified, 29 of which encoded known genes, 10 of which have undergone NCBI homology-based gene prediction and 15 of which encode hypothetical proteins (Fig. 3.6). The p140355 plasmid shared 99% sequence identity and 84% query cover with a plasmid previously detected in an MRSA isolate recovered in South Korea, pV030-8 (GenBank accession number: NC_010279). A ~7 kb insertion encoding *qacA* was present in p140355 but absent from pV030-8. The only two other *ileS2* and *qacA*-encoding plasmid sequences in the NCBI database (GenBank accessions numbers: KU882683 and KU882684) shared 50% and 24% query cover with p140355, respectively, and were both detected in *S. lugdunensis*. The read sets of the 51 MupR isolates which harboured *qacA*, all of which were recovered between 2014 and 2016, successfully mapped onto the p140355 sequence. The single MupR isolate which lacked *qacA* and was recovered in 2013, failed to map onto p140355.

Sub-cluster I isolates were particularly closely related, differing from each other by 0-80 SNVs (average: 26 SNVs; Fig. 3.3). The H1 isolates within sub-cluster I exhibited 0-70 pairwise SNVs and included isolates recovered in 11 different wards (Table 3.4). Isolates M14/0992 (MupS; screening isolate from H1) and M15/0213 (MupR; patient presented to GP), which were recovered from H1 HCWs, differed from the remaining sub-cluster I/H1 isolates by 12-53 SNVs and 7-47 SNVs, respectively. Sub-cluster I also included two isolate pairs which exhibited intra-pair mupirocin-susceptibility differences. In both instances, the MupR isolate was recovered after the MupS isolate, indicative of either selection *in vivo* for MupR MRSA or simultaneous carriage of MupR and MupS MRSA. Specifically, isolates M15/0148 (*ileS2*-negative and MupS) and M15/0637 (*ileS2*-positive and MupR), were recovered from patient P1 nine months apart and differed by 33 SNVs. Similarly, isolates, M15/0540 (*ileS2*-positive but MupS) and M15/0541 (*ileS2*-positive and MupR), were recovered from patient P2 23 days apart and differed by four SNVs (Fig. 3.3). The H1 environmental isolate differed from the remaining H1 isolates by 14-59 SNVs, indicating that environmental contamination could have contributed to the transmission of the outbreak strain in H1. Sub-cluster I/H1 isolates differed from sub-cluster I isolates recovered in other hospitals/HCFs by 0-77 SNVs. Isolate M15/0429, which was recovered from a patient who had been transferred directly from H1 to HCF-2, differed from the sub-cluster I/H1 isolates by 6-37 SNVs. The four community isolates within sub-cluster I differed from the sub-cluster I/H1 isolates and sub-cluster I isolates

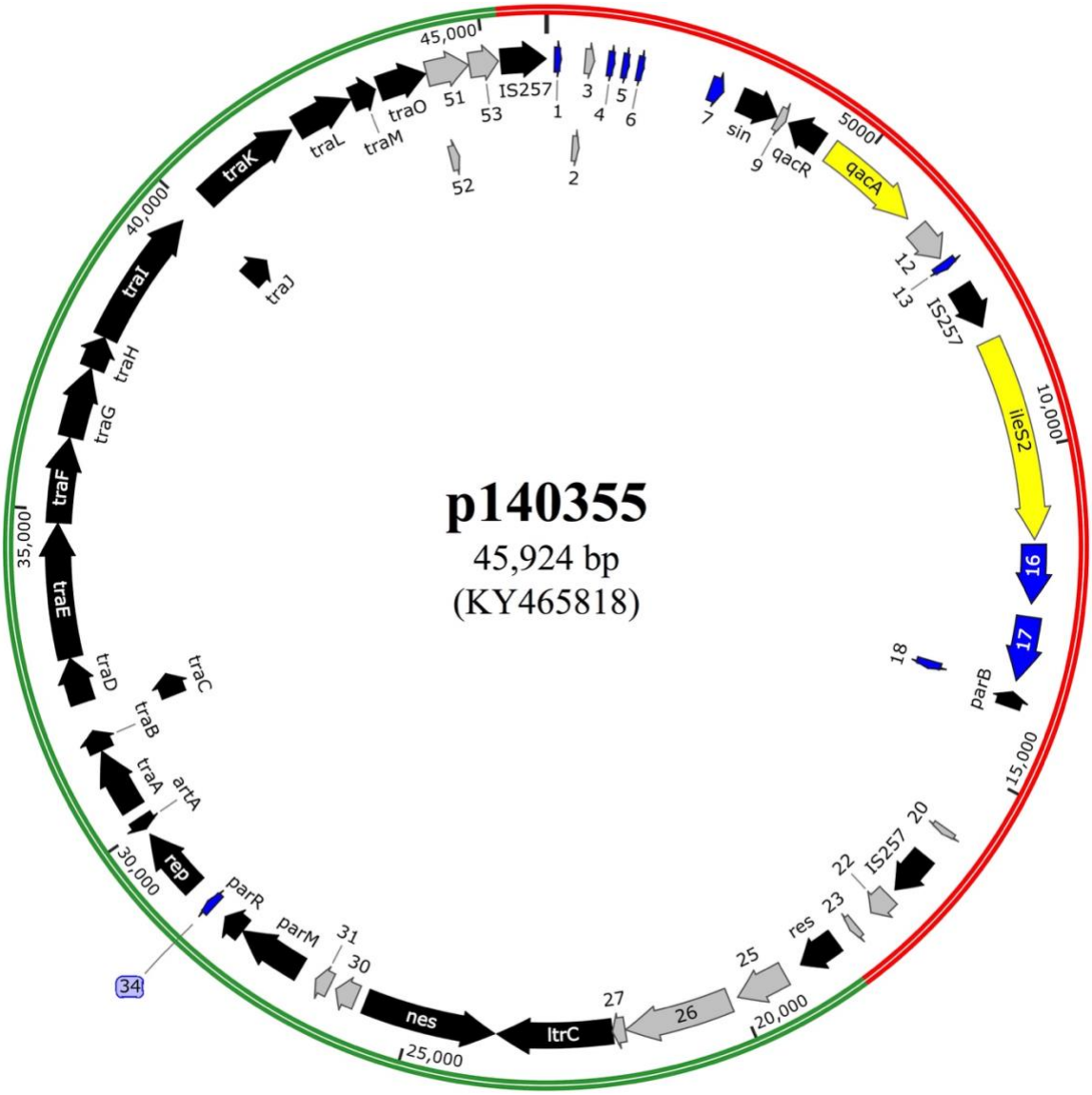


Figure 3.6 Genetic map of *S. aureus* multi-resistance plasmid, p140355 (GenBank accession number: KY465818). The plasmid backbone is shown in green, while the accessory region is depicted in red. All known genes are shown in black except for the antimicrobial resistance genes, which are highlighted in yellow. Genes which have undergone National Centre for Biotechnology Information prediction are shown in blue: 1, MobA/MobL family protein; 4, Fst family toxin; 5, quinone reductase; 6, quinone reductase; 7, MarR family transcriptional regulator; 13, haloacid dehalogenase-like hydrolase family protein; 16, fructosamine kinase family protein; 17, major facilitator superfamily protein; 18, DDE transposase superfamily protein; 34, XRE family transcriptional regulator. Genes encoding hypothetical proteins are shown in grey.

from other hospitals/HCFs by 3-80 and 5-67 SNVs, respectively. Community isolate M15/0637, which was recovered from a patient who had been recently discharged from H1, differed from the sub-cluster I/H1 isolates by 5-57 SNVs.

3.3.4 Sub-cluster II

Sub-cluster II isolates and intra-cluster outliers were recovered in six distinct geographic areas over a 36-month period (June 2013–June 2016; Fig. 3.4). This included the remaining (3/46) H1 isolates, and isolates recovered in H2 (2/4), H3 (1/5), H4 (2/3), H5 (2/2), H6 (2/3), H8 (1/1), H11 (1/1), H15 (1/1), HCF-1 (1/1) and the community (5/11; Fig. 3.3). All sub-cluster II isolates and one of the intra-cluster outliers (M16/0223) were recovered from patients (Table 3.4) The remaining intra-cluster outlier (M14/0597) was recovered from a H6 HCW (Table 3.4)

The sub-cluster II isolates exhibited similar phenotypic susceptibility and genotypic profiles to the sub-cluster I isolates. The vast majority (17/19) of sub-cluster II isolates and both intra-cluster outliers were identified as MDR, exhibiting phenotypic resistance to aminoglycosides, ß-lactams, MLS_B and/or tetracycline (Fig. 3.5). Accordingly, *aphA3*, *blaZ*, *erm*(C) and *tet*(K) were identified in sub-cluster II at high frequencies, as detailed in Table 3.5. None of the antimicrobial resistance genes detected in sub-cluster I at low frequencies were identified in sub-cluster II (Table 3.5). No sub-cluster II isolates exhibited mupirocin resistance or harboured the *ileS2* gene (Fig. 3.5; Table 3.5). As in sub-cluster I, DNA microarray profiling indicated that all sub-cluster II isolates were affiliated with WA MRSA-1/57.

Sub-cluster II isolates exhibited 2-127 pairwise SNVs (average: 93 SNVs; Fig. 3.6). Two isolates which were recovered on the same day from different patients on the same ward of hospital H5, differed by two SNVs, indicating recent intra-ward transmission. Isolate M16/0002, which was recovered in the community and isolate M16/0116, which was recovered in H8 43 days later, differed by six SNVs, suggesting recent transmission between H8 and the community. Apart from these instances, isolates within sub-cluster II differed from each other by 42-127 SNVs. The three H1 isolates in sub-cluster II differed by 42-121 SNVs. The intra-cluster outlier isolates, M14/0597 and M16/0223, differed from sub-cluster II isolates by 91-149 and 79-128 SNVs, respectively (Fig. 3.5).

3.3.5 Outliers

The 11 isolates outside of the major MST cluster (Fig. 3.2) were recovered over 29 months (October 2013–March 2016) in six geographically discrete regions (Fig. 3.4). These outliers were recovered in hospitals H2 (1/4), H6 (1/3), H7 (2/2), H9 (1/1), H10 (1/1), H12 (1/1), H14 (1/1) and H16 (1/1) and the community (2/11; Fig. 3.2). All outlier isolates were recovered from patients (Table 3.4)

Fusidic acid resistance, mediated by *fusC* (which is carried on SCC*fus*), was detected in 7/11 outliers and was the only phenotype that was statistically more common in the outliers than the cluster isolates (Fig. 3.5; Table 3.5). Indeed, aminoglycoside, MLS_B, mupirocin and tetracycline resistance were all statistically more common in the cluster isolates than in the outliers (Fig. 3.5). Accordingly, the outliers generally lacked the corresponding resistance genes (Table 3.5). However, the only instance of *tet*(M)-mediated tetracycline resistance was detected in an outlier isolate (M16/0123; Table 3.5). This was the only outlier which exhibited multi-drug resistance (Fig. 3.5). As in the major MST cluster, DNA microarray analysis indicated that all SCC*fus*-negative outliers were affiliated with WA MRSA-1/57. The SCC*fus*-positive outliers, however, were assigned to WA MRSA-1/45 (Coombs et al., 2011b).

Outlier isolates M14/0994 and M14/0886, which were recovered 49 days apart from patients on different hospital H7 wards, exhibited two SNVs, suggesting recent inter-ward transmission (Fig. 3.2). Excluding this instance, outlier isolates differed by 50-319 SNVs (average: 185 SNVs; Fig. 3.2).

3.4 Discussion

This study has confirmed the occurrence of a protracted CC1-MRSA-IV outbreak in a large Dublin hospital, indicated the existence of a conjugative *ileS2*- and *qacA*-encoding plasmid in the vast majority of outbreak isolates, and revealed the presence of the outbreak strain in six additional Irish hospitals/HCFs and the community. Furthermore, this investigation has revealed that the outbreak strain constitutes a MupR variant of the predominant and MDR CC1-MRSA-IV clone currently circulating throughout the healthcare system and community in Ireland.

Although the 89 MRSA isolates investigated generally exhibited the same ST, SCC*mec* type and *spa* type, cgMLST analysis permitted the identification of one large cluster and eleven outlier isolates. Furthermore, isolates within the major cluster were differentiated into two distinct sub-clusters, I and II, which exhibited average allelic distances of 25 and 61, respectively. An intra-host strain variation sub-study informed interpretation of these data by demonstrating that at any particular time, a single CC1-MRSA-IV strain could exhibit up to 17 allelic differences *in vivo*. Isolates within sub-cluster I were therefore considered recently transmitted, while those in sub-cluster II were considered closely related but generally not recently transmitted. Overall, these considerations indicated that the major MST cluster represented the predominant CC1-MRSA-IV clone in Ireland, while the detection of sub-cluster I confirmed the establishment of a protracted outbreak involving a largely MupR variant of this clone. The outbreak strain was detected in seven different hospitals/HCFs and the community, in the east/midlands of Ireland, while non-outbreak variants were identified in nine different hospitals/HCFs and the community, throughout the country (Fig. 3.3; Fig. 3.4). The detection of five community isolates in sub-cluster II, one of which was the earliest recovered cluster isolate, indicates it is possible that this clone emerged in the community prior to introduction into the healthcare system (Fig. 3.3; Table 3.4). This suggests that it may be appropriate to consider the community as an MRSA reservoir during targeted screening in Irish hospitals.

The considerable success of the outbreak strain was undoubtedly facilitated by its largely high-level MupR phenotype. Furthermore, the presence of *qacA* in the majority (53/57) of sub-cluster I isolates suggests that the outbreak strain was also typically resistant to chlorhexidine. Accordingly, the vast majority of attempts to decolonise patients harbouring this strain presumably failed and thus, it is likely that its dissemination was essentially unhindered. This highlights the necessity for further research into effective mupirocin and

chlorhexidine alternatives for decolonisation. While human, animal and *in-vitro* investigations have demonstrated the potential of multiple other agents in this regard, additional studies and large-scale clinical trials are required to further assess their efficacy (Poovelikunnel et al., 2015). For example, ethanol and manuka honey have been suggested as mupirocin alternatives (Poovelikunnel et al., 2017; Steed et al., 2014), while sodium hypochlorite has been proposed as a chlorhexidine substitute (Fritz et al., 2012). Significantly, seven MupS outbreak variants were also detected, demonstrating the unreliability of the method by which outbreak isolates were identified in H1 i.e. using high-level mupirocin resistance as a marker. It remains unclear whether the patients from whom these MupS isolates were recovered also harboured a MupR variant at the time of isolate recovery, highlighting the limitations associated with investigating one isolate per patient.

The *ileS2* and *qacA*-encoding plasmid detected in the majority (49/57) of outbreak isolates constitutes the first reported plasmid in *S. aureus* to encode resistance to the two antimicrobial agents (i.e. mupirocin and chlorhexidine) routinely used for *S. aureus* decolonisation (Fig. 3.6). While successful read alignment of the 49 *ileS2* and *qacA*-encoding outbreak isolates against the p140355 sequence suggested that these isolates harboured (a variant of) this plasmid, the earliest recovered outbreak isolate appeared to harbour a different *ileS2*-encoding plasmid, which did not encode *qacA*. Notably, however, PVL-negative CC1-MRSA-IV harbouring this undefined plasmid did not disseminate with the same success as those harbouring p140355. This suggests that the combination of mupirocin and chlorhexidine resistance (rather than mupirocin resistance alone) may have driven selection of p140355-harbouring MRSA in Ireland. However, it is possible that this was also influenced by other unknown contributory factors.

The radiation of the outbreak isolates from the centre of sub-cluster I suggests that a primary source transmitted the outbreak strain on multiple occasions (Fig 3.3). Interestingly, the close relatedness (7-53 SNVs) of the two H1 HCW isolates to the remaining H1 isolates demonstrates that HCWs cannot be ruled out as either the source of the outbreak, or a contributing factor to its persistence. However, in the absence of detailed information regarding HCW screening during the outbreak, their potential role cannot be determined. Following initiation of the outbreak, a complex transmission chain was established, as indicated by the highly branched network of isolates in sub-cluster I (Fig. 3.3). Several important routes of transmission were identified. Firstly, the presence of the

outbreak strain in 11 different H1 wards and its recovery from more than one patient in five of these wards, suggested that inter and intra-ward transmission occurred in H1, respectively (Table 3.4). Furthermore, the close relatedness (3-80 SNVs) of the outbreak isolates recovered in the community to those recovered in hospitals/HCFs, indicated that transmission between hospitals and the community also contributed to the dissemination of the outbreak strain. Indeed, a patient who harboured the outbreak strain while in H1 yielded an isolate (M15/0637) in the community nine months later, which differed from the H1 outbreak isolates by 5-57 SNVs. Finally, one instance of inter-hospital transmission was definitively identified when a patient who had been transferred from H1 to HCF-2 yielded an isolate (M15/0429) which differed from the H1 outbreak isolates by 6-37 SNVs. While it remains unclear how the outbreak strain was introduced into the remaining hospitals/HCFs in which it was detected, it is likely that this was caused by either staff movement between hospitals or HCFs (which is common in Ireland) and/or introduction directly from the community.

During the time period in which the predominant CC1-MRSA-IV clone circulated, the outlier isolates were recovered in eight different hospitals and the community, throughout Ireland (Table 3.4; Fig. 3.4). Although rarely MDR, the majority (7/11) of outliers harboured SCC*fus*, encoding *fusC*-mediated fusidic acid resistance. As indicated primarily by their MST positions, some outliers may constitute sporadic MRSA strains while others may represent a second CC1-MRSA-IV clone, which is not particularly prevalent in Ireland. Notably, however, DNA microarray profiling identified all isolates investigated as WA MRSA-1/45/57 and, considering their assignment to ST1, they perhaps better fit the description of WA MRSA-1 (rather than those of WA MRSA-45 or WA MRSA-57). The categorisation of the isolates in this way suggests that the outliers are neither sporadic nor clonally distinct from the cluster isolates, and contradicts the phenotypic, genotypic and WGS data generated during this investigation. Specifically, multidrug resistance (which is not generally associated with WA MRSA-1) was detected in the majority of cluster isolates, but in only one outlier (Fig. 3.5). Furthermore, the genotypic resistance profiles of the cluster isolates (which did not correspond with WA MRSA-1) differed considerably from those of the outliers (which often corresponded with WA MRSA-1). Finally, the cluster isolates were differentiated from the outliers by a sizeable allelic distance of 217. The identification of all 89 isolates as WA MRSA-1 therefore merits further investigation.

This investigation has revealed the high prevalence in Irish hospitals of a MDR PVL-negative CC1-MRSA-IV clone that was first detected in the community. Furthermore, an outbreak variant of this clone harbouring a novel *ileS2*- and *qacA*-encoding plasmid was detected in seven hospitals/HCFs and the community. This emphasises the need for further research into mupirocin and chlorhexidine alternatives and suggests that the community may constitute a significant source of MRSA strains that emerge in healthcare settings.

Chapter 4

The identification and characterisation of an emerging novel multidrug-resistant ST1-MRSA-IV-t127 clone which may have originated in South-Eastern Europe

4.1 Introduction

Reliable global surveillance of MRSA requires the development of standardised methods by which important clones can be identified. Significantly, however, the majority of MRSA clones which are now pandemic or endemic (including WA MRSA-1/45/57) were originally defined using various combinations of conventional typing techniques prior to the advent of WGS. However, as highlighted in Chapter 3, the extent to which these relatively imprecise typing methods can be relied upon for specific clone identification is now highly questionable. Accordingly, there often exists a degree of ambiguity in the literature regarding the global placement of strains identified during local surveillance investigations. This highlights the need for WGS-based phylogeny studies which can provide reference genomes, clarify lineage subtyping schemes and identify appropriate marker sequences for laboratories without WGS facilities.

As described in Chapter 1, section 1.4.1.1, useful marker sequences can be identified in the joining regions of particular SCC*mec* types. For example, SCC*mec* type IV can be identified as SCC*mec* type IVa through the detection of *cstB*-SCC2 (encoding a metallo-β-lactamase superfamily protein), in addition to the standard SCC*mec* IV markers (the class B *mec* complex and *ccrA*/*B2*). Similarly, SCC*mec* type IVa can be identified as MW2-like through the detection of *mvaS*-SCC (truncated 3-hydroxy-3-methylglutaryl CoA synthase) and *dcs*, in addition to the standard SCC*mec* IVa markers (Fig. 4.1) (Monecke et al., 2016b). Compared to the remaining 53 recently defined SCC*mec* subtypes (Chapter 1, section 1.5.6.1), MW2-like SCC*mec* IV is particularly widespread and has been detected in 12 different lineages (CC1, CC5, CC6, CC7, CC8, CC22, CC30, CC45, CC88, ST93, CC97 and ST617) to date (Monecke et al., 2016b). In addition to SCC*mec* subtyping, the identification of distinct SCC*mec* termini such as *dcs* is also relevant to the design of primers that span the SCC*mec* integration site i.e. from *orfX* in the *S. aureus* core genome into the terminus of SCC*mec*. This is significant due to the recent suggestion that integration site-spanning primers could be used in medical laboratories to distinguish between MRSA and *mecA*-positive staphylococci of lesser clinical relevance (Monecke et al., 2016b). To date, 15 different SCC*mec* termini have been described in detail, namely *dcs* and SCC termini 01-14 (Monecke et al., 2016b). Previous studies indicate that *dcs* (reference sequence: GenBank entry CP000046.1, nucleotide positions 34192-3437) is the most prevalent of all 15 SCC*mec* termini (Monecke et al., 2016b).

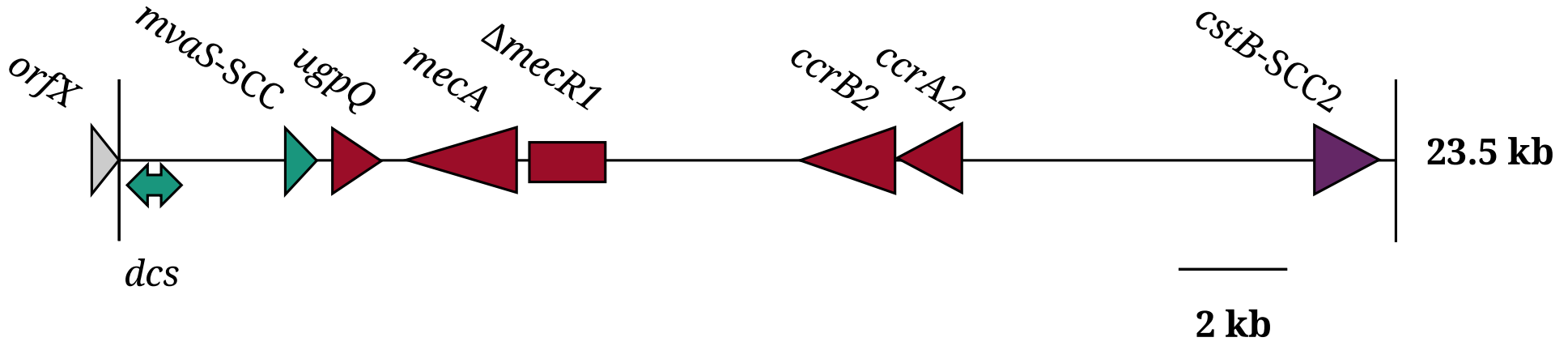


Figure 4.1 A schematic representation of the characteristic features of the MW2-like SCC*mec*. A recently proposed SCC*mec* subtyping scheme based one of 54 subtypes on the SCC*mec* element identified in the MRSA strain MW2 (PVL-positive ST1-MRSA-IV; GenBank accession number: BA000033.2) (Monecke et al., 2016b). The red features together constitute the defining characteristics of SCC*mec* IV among other SCC*mec* elements. The purple feature constitutes the defining characteristic of SCC*mec* IVa among other SCC*mec* IV elements. The green features together constitute the defining characteristics of MW2-like SCC*mec* IVa among other SCC*mec* IVa elements.

As detailed in Chapter 3, section 3.1, WA MRSA-1 does not typically exhibit multidrug resistance and is generally associated with resistance genes *blaZ* and *erm*(C), and variable *fusC* carriage. WA MRSA-1 is also associated with IEC genes *sak*, *scn* and *sea* (IEC type D), and enterotoxin genes *seh*, *sek* and *seq* (Coombs et al., 2011b). In the work described in the preceding chapter (Chapter 3), a combination of DNA microarray profiling and MLST indicated that all 89 PVL-negative CC1-MRSA-IV isolates recovered in Ireland between 2013 and 2016 were affiliated with the WA MRSA-1 clone. However, as outlined in Chapter 3, section 3.4, phenotypic, genotypic and WGS data indicated that while some of these isolates (i.e. some of the outliers) may indeed have corresponded to WA MRSA-1, the majority of isolates (i.e. the cluster) appeared to constitute a distinct clonal group. This part of the present study therefore aimed to investigate the origin of the CC1-MRSA-IV clone represented by the major cluster of isolates described in Chapter 3 in order to determine whether it constitutes a MDR sub-clone of WA MRSA-1 or a distinct PVL-negative CC1-MRSA-IV clone, yet to be formally defined.

4.2 Materials and Methods

4.2.1 Isolate selection

All 139 CC1-MRSA-IV isolates identified at the Irish NMRSARL between 2007 and 2017 were investigated in the present chapter. This included the 89 CC1-MRSA-IV isolates investigated in Chapter 3. The additional 50/139 isolates investigated in this chapter were identified as CC1-MRSA-IV based firstly on their exhibiting *spa* types corresponding to CC1 and secondly, on their harbouring SCC*mec* type IV, as determined by *in silico* DNA microarray profiling (see section 4.2.6). All available CC1-MSSA isolates identified at the NMRSARL (i.e. 10 isolates recovered between 2007 and 2017) were included for comparison to the Irish CC1-MRSA-IV isolates. These isolates were identified based on their exhibiting *spa* types corresponding to CC1. These 149 Irish isolates included two PVL-positive MRSA and three PVL-positive MSSA isolates, which were retained as potentially useful comparators isolates. Finally, 38 international *S. aureus* (28 MRSA and 10 MSSA) isolates were also included for comparison to the Irish CC1-MRSA-IV isolates. These isolates were selected from the global *S. aureus* database described in Chapter 2, section 2.6.6, based on their genotypic similarity to the Irish CC1-MRSA-IV, as determined by DNA microarray profiling (see section 4.2.7). Isolates were identified as *S. aureus* and MRSA/MSSA as described in Chapter 2, sections 2.3.2 and 2.3.3, respectively. Isolates were stored as described in Chapter 2, section 2.2.1.

4.2.2 *spa* typing

For the Irish isolates, traditional *spa* typing was performed as described in Chapter 2, section 2.4.3. All international isolates underwent *spa* typing following WGS (see below), as described in Chapter 2, section 2.6.4.

4.2.3 WGS

All isolates underwent WGS as described in Chapter 2, section 2.5.1 (Table 4.1).

4.2.4 *de novo* assembly

Sequence read sets were assembled into contigs as described in Chapter 2, section 2.6.2.

4.2.5 MLST

All isolates underwent traditional MLST using Ridom SeqSphere+ version 4.1 (Ridom GmbH), as described in Chapter 2, section 2.6.3.

Table 4.1 Whole-genome sequencing quality assurance data

Quality parameter	Average result
Trimmed read quality	36.3
N50[a]	429810.4
No. of contigs per isolate	22.4
Assembly coverage	161.8
% core-genome loci present[b]	99.5

[a]The minimum contig length required to cover half the genome.

[b]According to the previously defined core-genome multilocus sequence typing scheme (Leopold et al., 2014).

4.2.6 Genotyping and SCC*mec* subtyping using *in silico* DNA microarrays

All Irish isolates underwent genotyping and SCC*mec* subtyping using *in silico* DNA microarray profiling, as described in Chapter 2, section 2.6.5. To compare the complement of antimicrobial and virulence-associated genes between groups of isolates, the two-tailed Fisher's exact test was performed using the GraphPad QuickCalcs website: https://www.graphpad.com/quickcalcs/contingency1/ (accessed July 2018). Results were considered statistically significant if $p < 0.05$.

4.2.7 Selection of international isolates

This global database described in Chapter 2, section 2.6.6 includes 385 CC1-MRSA-IV isolates recovered in Europe, Australia, the Middle East, the USA and New Zealand. The vast majority (382/385; 99.2%) of these CC1-MRSA-IV isolates exhibit one of four main genotypic patterns, while those remaining exhibit unusual characteristics (such as SCC pseudoelements or composite SCC elements) and possibly represent sporadic strains or variants. Three of the four main CC1-MRSA-IV patterns each match the description of previously defined clones (Monecke et al., 2011). These clones are (i) PVL-negative strains with SCC*mec* IV or SCC*mec* IV+SCC*fus* elements, referred to as WA MRSA-1/45/57, (ii) the PVL-positive "USA400" clone and (iii) a PVL-positive strain with a SCC*mec* IV+SCC*fus* composite element. A fourth pattern can be defined that differs from any well-defined CC1-MRSA-IV clone in several markers (see results section of this chapter). This pattern matches those of both the MDR CC1-MRSA-IV clone identified in Chapter 3 and a group of isolates previously recovered in Romania (Monecke et al., 2014). Hereafter, this fourth genotypic pattern is referred to as the "undefined" pattern or clone.

Following *in silico* genotyping, the Irish isolates could be compared to those represented in the global database. The vast majority of Irish MRSA isolates investigated exhibited either the WA MRSA-1/45/57 or the undefined genotypic pattern. Representative (based on country/location of isolation, genotypic variations and/or date of recovery) selections of international MRSA isolates exhibiting each of these two genotypic patterns were selected from the global strain collection for further analysis using WGS. Specifically, one German and two UAE CC1-MRSA-IV isolates exhibiting the WA MRSA-1/45/57 genotypic pattern, and a further nineteen German and five Romanian CC1-MRSA-IV isolates exhibiting the undefined genotypic pattern were selected. A representative selection of international MSSA isolates exhibiting the undefined genotypic pattern (minus the SCC*mec* genes) was also selected from the global database for further analysis using

WGS. This included 10 Romanian CC1-MSSA isolates. Importantly, it was noted that under-resourced countries were often poorly represented in the strain collection.

4.2.8 Phylogenetic analysis

Whole-genome sequencing data were analysed using the wgMLST scheme available in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium), as described in Chapter 2, section 2.6.8. A multiple sequence alignment of the concatenated core genes and a distance matrix based on cgSNVs was generated. Core-genome SNVs were called exclusively in positions shared by all samples. Only cgSNVs with at least 5x read coverage (including 1x coverage in each direction) were considered. Potentially indel-related cgSNVs, occurring within 12 bp of each other, were removed. Positions with ambiguous base calls and cgSNVs in repetitive regions were excluded. Two separate NJTs were generated based on this cgSNV analysis, as described in Chapter 2, section 2.6.10. The first NJT included all MRSA isolates only. This tree was used to confirm the clonality of the isolates exhibiting the undefined genotypic pattern. The second NJT included all MRSA and MSSA isolates. This tree was used to determine whether any of the MSSA isolates investigated were closely related to the undefined clone.

4.2.9 Enhanced SCC*mec* analysis

Specific SCC*mec* alleles of interest were compared between isolates using Clustal Omega (Sievers et al., 2011). Specific SCC*mec* regions of interest were investigated using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), annotated using BioNumerics v7.6 (Applied Maths) and drawn using Lucid Chart (https://www.lucidchart.com). The contig sets of a relevant selection isolates were aligned against the complete SCC*mec* sequence of a single isolate (which had been obtained on a single contig) using the Burrows-Wheeler aligner (http://arxiv.org/abs/1303.3997). Artemis sequence viewer (https://www.sanger.ac.uk/science/tools/artemis) was used to visually assess the mapping of contigs.

4.2.10 Accession numbers

The contigs of a representative CC1-MRSA-IV isolate exhibiting the undefined genotypic pattern (clade A; isolate A_01) were submitted to GenBank (accession number: RBVO00000000.1). The sequence read sets of all isolates investigated were submitted to the NCBI Sequence Read Archive database (accession number: PRJNA494507).

4.3 Results

4.3.1 Description of isolates investigated

Upon phylogenetic analysis of the 167 MRSA isolates only, two main clades (one large and one small) were identified (Fig. 4.2). The large clade included the Chapter 3 cluster isolates, while the small clade included the Chapter 3 outliers. A tight subclade of 86 isolates was identified within the large clade (Fig. 4.2). This subclade included the Chapter 3 outbreak isolates. Considering the aim of the present chapter (section 4.1), and that none of the international MRSA isolates grouped within the tight subclade, a single centrally-located subclade isolate (isolate A_68; Table 4.2) was selected as an outbreak representative for all further analyses. A total of 82 MRSA (54 Irish and 28 international) and 20 MSSA (10 Irish and 10 international) isolates were therefore examined in detail during this chapter. The epidemiological information associated with these 102 isolates is detailed in Table 4.2.

The vast majority (79/82) of MRSA isolates were identified as ST1 (allelic profile: 1-1-1-1-1-1-1). The remaining three MRSA isolates were identified as ST4911 (1-1-1-1-1-1-649), ST4913 (1-1-1-1-1-1-648) and ST4914 (1-1-663-1-1-1-1). All MRSA isolates harboured SCC*mec* type IVa and exhibited *spa* type t127 (*spa* repeat successions: t127, 07-23-21-16-34-33-13). The majority (18/20) of MSSA isolates were identified as ST1, while the two remaining isolates were identified as ST4910 (1-1-1-1-40-1-1) and ST4912 (1-731-1-1-1-1-1). The majority (18/20) of MSSA isolates were also identified as t127, while the remaining two isolates were characterised as t5633 (15-13) and t18248 (07-23-21-16-34-33-20). All non-ST1 STs identified were ST1 SLVs which differed from ST1 at just one nucleotide position.

4.3.2 Identification of four distinct clades using cgSNV analysis

A total of 2,891 core-genome nucleotide positions exhibited variations that fulfilled all filtering criteria. Neighbour-joining tree analysis based on these 2,891 positions revealed that the 102 isolates investigated grouped into one of four main clades (A, B, C and D; Fig. 4.3). Clade A included 58 MRSA isolates and 10 MSSA isolates. Clade B included 23 MRSA isolates, 20 of which formed a loose subclade termed subclade B1, and six MSSA isolates. Clade C included one MRSA and two MSSA isolates, while clade D included two MSSA isolates only.

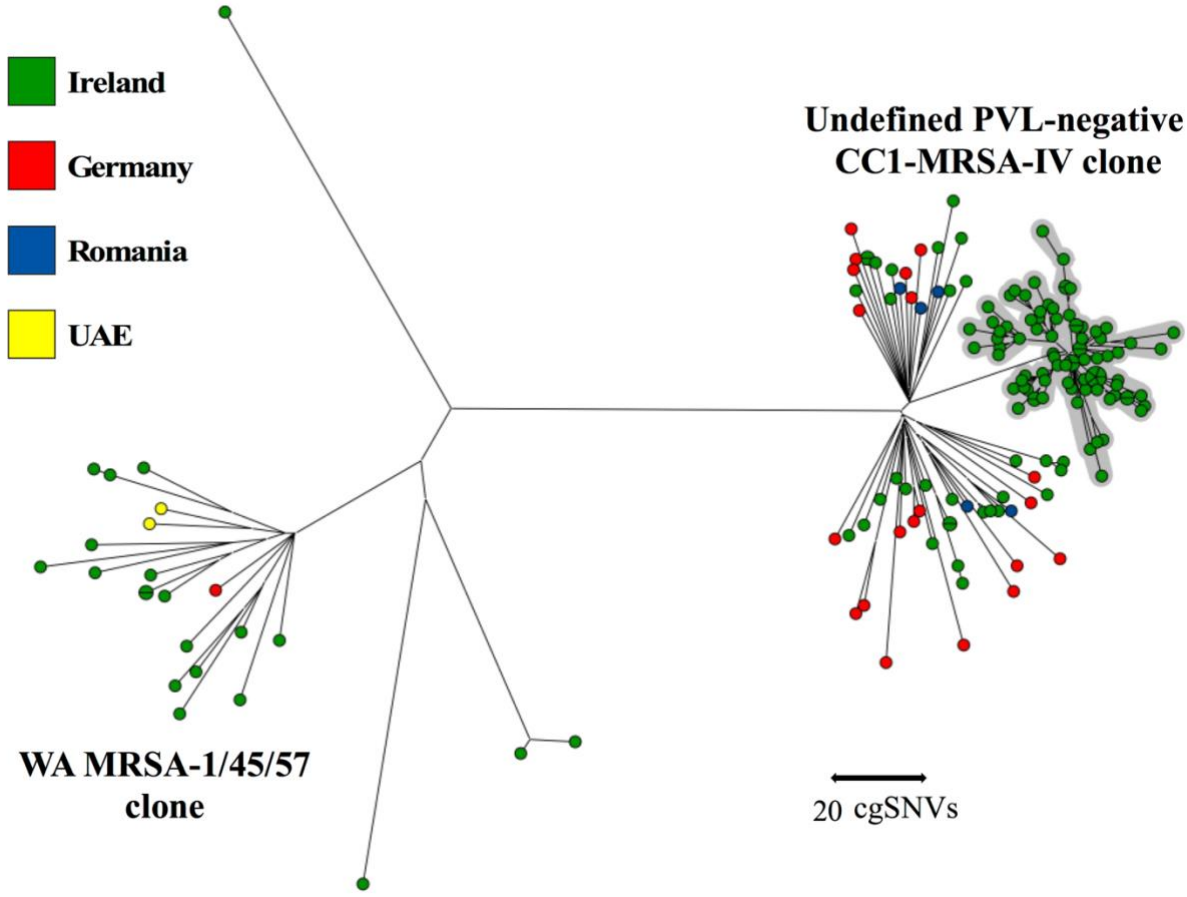


Figure 4.2 A neighbour-joining tree based on a cgSNV analysis of 167 CC1-MRSA-IV isolates. The countries in which the isolates were recovered are indicated in the colour legend. The outbreak cluster is shaded in grey. All branches yielded 100% permutation resampling support. Abbreviations: cgSNV, core-genome single nucleotide variation.

Table 4.2 The epidemiological data associated with 82 CC1-MRSA-IV and 20 CC1-MSSA isolates recovered in Ireland, Germany, Romania and the UAE, between 2007 and 2018

Isolate[a]	Year/date of recovery	Country of recovery	MRSA/ MSSA	Source[b]	Recovery site
Clade A					
A_01	20/12/2007	Ireland	MRSA	Community	Unknown
A_02	13/01/2008	Ireland	MRSA	Community	Unknown
A_03	24/07/2012	Ireland	MRSA	IRL_08	Unknown
A_04	24/09/2012	Ireland	MRSA	IRL_03	Unknown
A_05	12/10/2012	Ireland	MRSA	Community	Unknown
A_06	01/03/2013	Ireland	MRSA	IRL_01	Colonisation
A_07	13/06/2013	Ireland	MRSA	Community	Infection
A_08	25/02/2014	Ireland	MRSA	IRL_01	Colonisation
A_09	01/05/2014	Ireland	MRSA	IRL_14	Colonisation
A_10	10/05/2014	Ireland	MRSA	IRL_03	Unknown
A_11	14/07/2014	Ireland	MRSA	IRL_08	Unknown
A_12	23/09/2014	Ireland	MRSA	IRL_11	Colonisation
A_13	23/09/2014	Ireland	MRSA	IRL_11	Colonisation
A_14	13/01/2015	Ireland	MRSA	IRL_03	Unknown
A_15	25/01/2015	Ireland	MRSA	IRL_01	Colonisation
A_16	24/03/2015	Ireland	MRSA	Community	Unknown
A_17	01/04/2015	Ireland	MRSA	Community	Infection
A_18	30/04/2015	Ireland	MRSA	IRL_05	Unknown
A_19	11/05/2015	Ireland	MRSA	IRL_02	Unknown
A_20	14/05/2015	Ireland	MRSA	IRL_06	Unknown
A_21	17/06/2015	Ireland	MRSA	Community	Infection
A_22	18/06/2015	Ireland	MRSA	IRL_07	Unknown
A_23	13/07/2015	Ireland	MRSA	IRL_12	Unknown
A_24	24/12/2015	Ireland	MRSA	IRL_08	Infection
A_25	05/02/2016	Ireland	MRSA	IRL_06	Colonisation
A_26	31/03/2016	Ireland	MRSA	IRL_13	Infection
A_27	16/07/2016	Ireland	MRSA	IRL_05	Unknown
A_28	23/12/2016	Ireland	MRSA	IRL_05	Unknown
A_29	02/03/2017	Ireland	MRSA	IRL_08	Unknown
A_30	06/03/2017	Ireland	MRSA	IRL_08	Colonisation
A_31	07/03/2017	Ireland	MRSA	IRL_08	Colonisation
A_32	16/05/2017	Ireland	MRSA	IRL_08	Unknown
A_33	2009	Romania	MSSA	RM_1	Infection
A_34	2010	Romania	MRSA	RM_1	Infection
A_35	2010	Romania	MRSA	RM_1	Infection
A_36	2010	Romania	MSSA	RM_1	Infection

Table 4.2 continued overleaf

Isolate[a]	Year/date of recovery	Country of recovery	MRSA/ MSSA	Source[b]	Recovery site
Clade A continued...					
A_37	2010	Romania	MSSA	RM_1	Infection
A_38	2010	Romania	MRSA	RM_1	Infection
A_39	2010	Romania	MSSA	RM_1	Infection
A_40	2010	Romania	MSSA	RM_1	Infection
A_41	2011	Romania	MSSA	RM_1	Infection
A_42	2011	Romania	MSSA	RM_1	Infection
A_43	2012	Romania	MSSA	RM_1	Infection
A_44	2012	Romania	MSSA	RM_1	Infection
A_45	2012	Romania	MSSA	RM_1	Colonisation
A_46	2012	Romania	MRSA	RM_1	Colonisation
A_47	Unknown	Romania	MRSA	RM_1	Unknown
A_48	2016	Germany	MRSA	GR_1	Unknown
A_49	2016	Germany	MRSA	GR_2	Colonisation
A_50	2016	Germany	MRSA	GR_2	Colonisation
A_51	2016	Germany	MRSA	Community	Colonisation
A_52	2016	Germany	MRSA	GR_3	Colonisation
A_53	2016	Germany	MRSA	GR_3	Colonisation
A_54	2016	Germany	MRSA	GR_3	Colonisation
A_55	2016	Germany	MRSA	GR_3	Infection
A_56	2017	Germany	MRSA	GR_2	Colonisation
A_57	2017	Germany	MRSA	GR_2	Infection
A_58	2017	Germany	MRSA	GR_2	Colonisation
A_59	2017	Germany	MRSA	GR_3	Colonisation
A_60	2017	Germany	MRSA	GR_3	Colonisation
A_61	2017	Germany	MRSA	GR_3	Infection
A_62	2017	Germany	MRSA	GR_3	Colonisation
A_63	2017	Germany	MRSA	GR_3	Infection
A_64	2017	Germany	MRSA	GR_2	Colonisation
A_65	2017	Germany	MRSA	GR_2	Colonisation
A_66	2018	Germany	MRSA	GR_3	Colonisation
A_67	2018	Germany	MRSA	GR_2	Colonisation
A_68	30/05/2014	Ireland	MRSA	IRL_01	Infection
Clade B1					
B1_01	09/08/2012	Ireland	MRSA	IRL_15	Unknown
B1_02	18/01/2013	Ireland	MRSA	IRL_16	Unknown
B1_03	25/03/2013	Ireland	MRSA	IRL_03	Unknown
B1_04	09/08/2013	Ireland	MRSA	IRL_03	Unknown
B1_05	25/07/2014	Ireland	MRSA	Community	Infection
B1_06	30/10/2014	Ireland	MRSA	IRL_17	Unknown

Table 4.2 continued overleaf

Isolate[a]	Year/date of recovery	Country of recovery	MRSA/ MSSA	Source[b]	Recovery site
Subclade B1 continued…					
B1_07	27/11/2014	Ireland	MRSA	IRL_15	Infection
B1_08	17/12/2014	Ireland	MRSA	IRL_17	Unknown
B1_09	28/01/2015	Ireland	MRSA	IRL_18	Unknown
B1_10	28/01/2015	Ireland	MRSA	IRL_18	Colonisation
B1_11	06/02/2015	Ireland	MRSA	IRL_19	Colonisation
B1_12	19/06/2015	Ireland	MRSA	IRL_13	Unknown
B1_13	14/10/2015	Ireland	MRSA	IRL_20	Unknown
B1_14	02/03/2016	Ireland	MRSA	Community	Infection
B1_15	08/03/2016	Ireland	MRSA	IRL_03	Unknown
B1_16	15/12/2016	Ireland	MRSA	IRL_01	Unknown
B1_17	29/05/2017	Ireland	MRSA	IRL_21	Unknown
B1_18	2007	Germany	MRSA	GR_1	Infection
B1_19	2009	UAE	MRSA	UE_1	Unknown
B1_20	2009	UAE	MRSA	UE_1	Unknown
Remaining clade B					
B_01	20/10/2004	Ireland	MSSA	IRL_08	Unknown
B_02	18/01/2010	Ireland	MSSA	IRL_17	Unknown
B_03	24/10/2013	Ireland	MRSA	IRL_23	Infection
B_04	20/08/2014	Ireland	MSSA	IRL_03	Unknown
B_05	03/11/2015	Ireland	MSSA	IRL_01	Unknown
B_06	08/01/2016	Ireland	MRSA	IRL_13	Unknown
B_07	18/10/2016	Ireland	MRSA	IRL_03	Unknown
B_08	26/02/2017	Ireland	MSSA	IRL_03	Unknown
B_09	21/05/2017	Ireland	MSSA	IRL_01	Infection
Clade C					
C_01	31/07/2013	Ireland	MSSA	IRL_19	Unknown
C_02	28/10/2013	Ireland	MSSA	IRL_22	Unknown
C_03	18/04/2016	Ireland	MRSA	IRL_21	Unknown
Clade D					
D_01	15/03/2017	Ireland	MSSA	IRL_08	Colonisation
D_02	15/03/2017	Ireland	MSSA	IRL_08	Colonisation

[a]Isolates are grouped according to their position in the core-genome single nucleotide variation-based neighbour-joining tree generated in this part of the present study. All isolates in bold were included in the work presented in Chapter 3.

[b]Isolates sourced from the community were defined as those recovered in a general medical practitioner's surgery, an emergency department or an outpatient's department. All other isolates were associated with specific hospitals which are indicated using a two-letter country code (GR, Germany; IRL, Ireland; RM, Romania; UE, United Arab Emirates) and a number.

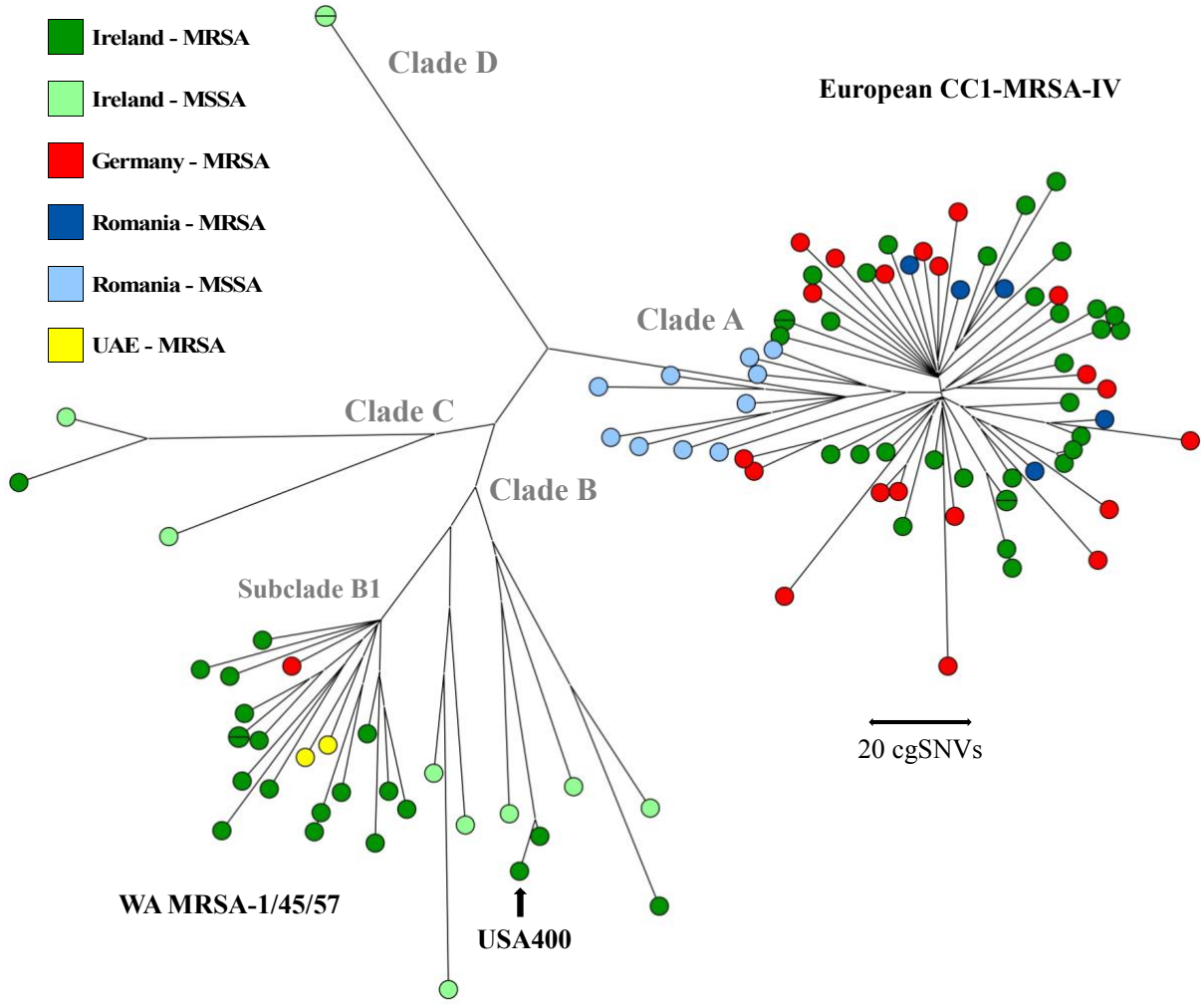


Figure 4.3 A neighbour-joining tree based on a cgSNV analysis of 82 CC1-MRSA-IV and 20 CC1-MSSA isolates. The countries in which the isolates were recovered are indicated in the colour legend. All branches yielded 100% permutation resampling support. Abbreviations: cgSNV, core-genome single nucleotide variation.

The clade A MRSA isolates exhibited the undefined CC1-MRSA-IV genotypic pattern, while the subclade B1 isolates exhibited the WA MRSA-1/45/57 genotypic pattern. The clade A MRSA isolates were therefore characterised in detail and compared to the subclade B1 isolates. The remaining isolates in clade B and those in clades C and D were examined separately. Clade A and subclade B1 were differentiated by 171 cgSNVs, while clade A MRSA specifically were differentiated from subclade B1 by 188 cgSNVs.

4.3.3 Identification of clade specific SCC*mec* characteristics

The SCC*mec* subtyping array indicated that all MRSA isolates in both clade A and subclade B1 harboured MW2-like SCC*mec* IVa. In clade A only, however, the *dcs* sequence was not detected. Fortunately, the entire SCC*mec* element of one clade A isolate (A_51; Table 4.2) was obtained on a single contig, facilitating its detailed examination. The contig sets of the remaining cluster isolates aligned well to this sequence. A previously undefined SCC*mec* terminus termed SCC terminus 15 (nucleotide positions 280690 – 280912 of GenBank entry RBVO000005.1), was subsequently identified (Fig. 4.4). Furthermore, five ORFs (nucleotide positions 289013 – 285400 of GenBank entry RBVO000005.1) which are absent from MW2-like SCC*mec*, were identified adjacent to SCC terminus 15 (Fig. 4.4). This ~4.7 kb insertion (i.e. SCC terminus 15 plus the five ORFs) was also identified adjacent to *orfX* in the previously sequenced SCC*mec* elements of six ST8/ST5 MRSA strains (GenBank accession numbers: CP007672.1, CP007670.1, HM030720.1, HF937103.1, KF184643.1 and KF234240.1), five *S. epidermidis* strains (LT571449.1, JHQC01000015.1, AKHK01000038.1, HG813242.1 and AKGT01000059.1) and one *Staphylococcus capitis* strain (CP007601.1). Two ORFs (A and B) located adjacent to *dcs* in MW2-like SCC*mec* were absent from the clade A SCC*mec* element (Fig. 4.4).

All clade A MRSA isolates harboured the same allelic variants of the cassette chromosome recombinase genes, *ccrA2* (1350 bp) and *ccrB2* (1629 bp). All subclade B1 isolates harboured the same *ccrA2* allele, and the vast majority (16/20) harboured the same *ccrB2* allele, while those remaining harboured an allele which differed from the predominant *ccrB2* allele at 1 nucleotide position. The clade A and subclade B1 *ccrA2* alleles differed by 23 SNVs, while the clade A *ccrB2* allele differed from the predominant subclade B1 *ccrB2* allele by 51 SNVs. In addition to MW2-like SCC*mec*, half (10/20) of the clade subclade B1 isolates also harboured a SCC*fus* element including the SCC_{476}-marker gene, *tirS*.

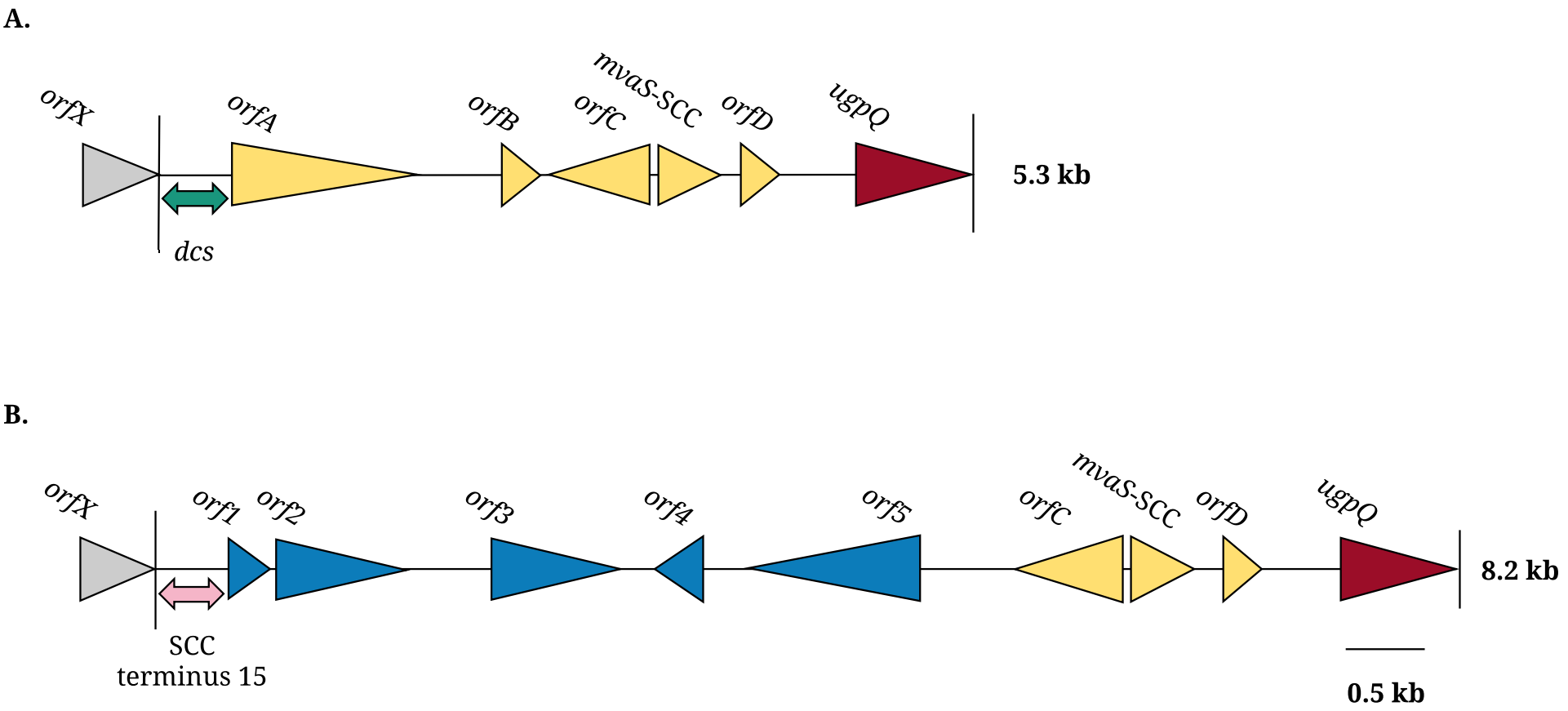


Figure 4.4 A schematic representation of the J3 regions of (A) MW2 SCC*mec* and (B) the SCC*mec* element identified in the clade A MRSA isolates. A recently proposed SCC*mec* subtyping scheme based one of 54 SCC*mec* subtypes on the SCC*mec* element identified in the MRSA strain MW2 (PVL-positive ST1-MRSA-IV; GenBank accession number: BA000033.2) (Monecke et al., 2016b). The SCC*mec* element identified in the clade A MRSA isolates encoded all the MW2-like SCC*mec* marker sequences except *dcs*. A 4,710-nucleotide insertion encoding five ORFs and a novel SCC*mec* terminus (termed SCC terminus 15) was subsequently identified.

4.3.4 Clade A

4.3.4.1 MRSA

The 58 clade A MRSA isolates were recovered in Ireland (n = 33), Germany (n = 20) and Romania (n = 5), and exhibited 0-109 (average 62.6, standard deviation [SD] 13.8) pairwise cgSNVs (Fig. 4.3). All isolates were identified as ST1-MRSA-IVa-t127, with the exception of a single ST4911-MRSA-IVa-t127 isolate from Germany (Table 4.3). The 33 Irish MRSA isolates were recovered in 11 different hospitals/HCFs and the community between 2007 and 2017 (Table 4.3). The first two clade A MRSA isolates identified in Ireland, A_01 and A_02, were recovered in the community in 2007 and 2008, respectively (Table 4.2). These isolates were recovered 26 days apart and differed by 52 cgSNVs. The third clade A MRSA isolate identified in Ireland, A_03, was recovered in a hospital in 2012 (Table 4.2). The German isolates were recovered between 2016 and 2018, and included 1/5 isolates from a hospital in Saxony (i.e. there were five isolates from a Saxon hospital in the global database that exhibited the undefined array pattern; one of these underwent WGS and grouped into clade A) and 19/37 isolates recovered in two different hospitals and the community in the Bavarian city of Regensburg (Table 4.3). Similarly, clade A included 5/40 Romanian isolates recovered in a hospital in the north-eastern city of Iaşi between 2010 and 2012 (Table 4.3). No phylogenetic subgrouping of isolates recovered in the same country was observed (Fig. 4.3). Indeed, the Irish isolates exhibited 1-102 (average 69.0, SD 14.0) pairwise cgSNVs, the German isolates exhibited 12-115 (average 79.0, SD 16.2) pairwise cgSNVs and the Romanian isolates exhibited 53-71 (average 59.0, SD 6.8) pairwise cgSNVs.

The MRSA isolates within clade A harboured a broader range of antimicrobial resistance genes than those in subclade B1 (Table 4.4). Specifically, *aphA3*, *aadE* and *sat* were detected in all clade A MRSA but were universally absent from subclade B1. Furthermore, *erm*(C) and *tet*(K) were significantly ($p < 0.0001$) more common in MRSA in clade A than in subclade B1. The majority (47/58) of clade A MRSA isolates harboured the IEC type E (*sak* and *scn*), while those remaining (11/58) lacked IEC-associated genes and harboured an undisrupted *hlb* gene. None of the clade A MRSA isolates harboured any enterotoxin genes other than *seh*, which is universally present in CC1.

4.3.4.2 MSSA

Considering that clade A included MRSA isolates recovered in Ireland and Romania as early as 2007 and 2010, respectively, CC1 MSSA from both Ireland and Romania were

Table 4.3. Epidemiological and genotypic data associated with 82 CC1-MRSA-IV and 20 CC1-MSSA isolates recovered in Ireland, Germany, Romania and the UAE, between 2007 and 2018.

NJT position[a]	Country	MRSA/ MSSA	No. of isolates	Recovery period	Source(s)	Sequence type(s)[b]	*spa* type(s)[c]	SCC element subtypes
Clade A	Ireland	MRSA	33	2007-2017	11 hospitals/HCFs Community	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Germany	MRSA	20	2016-2018	3 hospitals Community	ST1(*n* = 19) ST4911 (*n* = 1)	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Romania	MRSA	5	2010-2012	1 hospital	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
		MSSA	10	2009-2012	1 hospital	ST1 (*n* = 8) ST4910 (*n* = 1) ST4912 (*n* = 1)	t127 (*n* = 8) t5633 (*n* = 1) t18248 (*n* = 1)	SCC-negative
Subclade B1	Ireland	MRSA	17	2012-2017	9 hospitals Community	ST1 (*n* = 15) ST4913 (*n* = 1) ST4914 (*n* = 1)	t127	MW2-like SCC*mec* IVa (*n* = 9) SCC*mec* IVa/SCC*fus*$_{476}$ (*n* = 8)
	UAE	MRSA	2	2009	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Germany	MRSA	1	2007	1 hospital	ST1	t127	MW2-like SCC*mec*
Remaining clade B	Ireland	MRSA	3	2014-2016	3 hospitals	ST1	127	MW2-like SCC*mec* IVa (*n* = 1) SCC*mec* IVa/SCC*fus*$_{476}$ (*n* = 2)
		MSSA	6	2004-2017	4 hospitals	ST1	127	SCC*fus*$_{476}$
Clade C	Ireland	MRSA	1	2016	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Ireland	MSSA	2	2013	2 hospitals	ST1	127	SCC*fus*$_{476}$
Clade D	Ireland	MSSA	2	2017	1 hospital	ST1	127	SCC-negative

Footnotes overleaf

[a]Based on core-genome single nucleotide variation analysis.

[b]Multilocus sequence typing was performed using Ridom SeqSphere+ version 4.1 (Ridom GmbH). Allelic profiles: ST1, 1-1-1-1-1-1-1; ST4910, 1-1-1-1-40-1-1; ST4911, 1-1-1-1-1-1-649; ST4912, 1-731-1-1-1-1-1; ST4913, 1-1-1-1-1-1-648; ST4914, 1-1-663-1-1-1-1.

[c]*spa* typing was performed either using Ridom SeqSphere+ version 4.1, or as previously described in Chapter 2, section 2.4.3. *spa* repeat successions: t127, 07-23-21-16-34-33-13; t922, 07-23-21-16-33-13; t5633, 15-13; t18248, 07-23-21-16-34-33-20.

Abbreviations: HCFs, healthcare facilities; NJT, neighbour-joining tree; ST, sequence type.

Table 4.4 Differences in resistance and virulence-associated gene carriage between CC1-MRSA-IVa isolates in clade A and subclade B1

Gene	Clade A[a]		Subclade B1[a]		
	n	**%**	***n***	**%**	***p* value[a]**
Resistance					
aadD	0	0	**3**	**15**	**0.02**
aadE	**58**	**100**	0	0	**<0.0001**
aacA-aphD	2	3	0	0	1
aphA3	**58**	**100**	0	0	**<0.0001**
blaZ	58	100	20	100	1
erm(A)	0	0	**4**	**20**	**0.0034**
erm(C)	**57**	**98**	3	15	**<0.0001**
fusB	0	0	0	0	NA
fusC	0	0	**10**	**50**	**<0.0001**
ileS2	1	2	0	0	1
lnu(A)	0	0	0	0	NA
mecA	58	100	20	100	1
qacA	2	3	0	0	1
sat	**58**	**100**	0	0	**<0.0001**
tet(K)	**53**	**91**	0	0	**<0.0001**
Virulence					
hlb[b]	11	19	0	0	0.06
sak	47	81	20	100	0.06
scn	47	81	20	100	0.06
sea	0	0	**19**	**95**	**<0.0001**
seh	58	100	20	100	1
sek	0	0	**19**	**95**	**<0.0001**
seq	0	0	**19**	**95**	**<0.0001**

[a]Statistically significant results are shown in bold.

[b]Undisrupted-*hlb*

investigated as potential precursors to clade A MRSA (Table 4.3). Forty Romanian CC1-MSSA isolates exhibiting the undefined CC1-MRSA-IV genotypic pattern (excluding genes typically located in SCC*mec*) were identified in the global database. Ten of these isolates were selected for WGS and subsequent cgSNV analysis. They grouped into clade A, differing from the MRSA isolates within this clade by 47-130 cgSNVs (Fig. 4.3). The Romanian MSSA isolates were recovered in the same hospital as the Romanian ST1-MRSA-IV isolates. The majority (8/10) of Romanian MSSA isolates were characterised as ST1 MSSA, while two isolates were identified as ST4910 and ST4912 (Table 4.3). Similarly, the majority (8/10) of Romanian MSSA isolates were identified as *spa* type t127, while the ST4910 isolate was identified as *spa* type t5633 and one ST1 isolate was assigned *spa* type t18248 (Table 4.3). Similar to clade A MRSA, 8/10 clade A MSSA isolates carried *aphA3*, *aadE* and *sat*. The *erm*(C) gene was also detected in 8/10 isolates, while 9/10 isolates harboured *tet*(K). Furthermore, clade A MSSA did not carry any enterotoxin genes other than *seh*, and either harboured IEC type E (8/10) or lacked IEC associated genes (2/10). Importantly, none of the 10 Irish MSSA isolates investigated grouped in close proximity to the clade A MRSA isolates (Fig. 4.3).

4.3.5 Subclade B1 (MRSA only)

The subclade B1 isolates were recovered in Ireland (*n* = 17), the UAE (*n* = 2) and Germany (*n* = 1), and differed by 0-116 (average 77.9, SD 17.0) pairwise cgSNVs (Fig. 4.3). All subclade B1 isolates were identified as t127 (Table 4.3). The Irish isolates were recovered in nine different hospitals and the community between 2012 and 2017 (Table 4.3). Eight of the Irish isolates were identified as ST1-MRSA-IVa/SCC*fus*$_{476}$, while seven were identified as ST1-MRSA-IVa and the remaining two were characterised as ST4913-MRSA-IVa and ST4914-MRSA-IVa. The UAE isolates included 2/4 isolates recovered in a hospital in Abu Dhabi in 2009, both of which were characterised as ST1-MRSA-IVa/SCC*fus*$_{476}$. The German isolate represented a group of four isolates recovered in the aforementioned Dresden hospital in 2007 and was identified as ST1-MRSA-IVa. A large group of Australian isolates (*n* = 46; recovered between 2001 and 2009) exhibiting WA MRSA-1/45/57 genotypic patterns were also identified in the global database. However, these Australian isolates did not undergo WGS as part of this study.

The *fusC*, *aadD*, and *erm*(A) genes were the only resistance genes that were more common in subclade B1 than in clade A MRSA (Table 4.4). However, the subclade B1 isolates harboured a wider range of virulence-associated genes than clade A MRSA (Table 4.4).

Specifically, in addition to the CC1-associated *seh* gene, the enterotoxin genes *sea*, *sek* and *seq* were significantly common in subclade B1 (Table 4.4). The majority (19/20) of subclade B1 isolates harboured IEC type D (*sea*, *sak* and *scn*), while IEC type E (*sak* and *scn*) was detected in a single instance.

4.3.6 Remaining isolates

All remaining isolates were recovered in Ireland (Fig. 4.3; Table 4.3). The nine remaining (non-subclade B1) clade B isolates differed from those in subclade B1 by 83-158 cgSNVs. These isolates included two closely related (10 cgSNVs) PVL-positive ST1-MRSA-IVa-t127 isolates, one of which was identified as USA400 based on its genotypic pattern and the other of which harboured SCC*fus*$_{476}$ (Fig. 4.3). Clade B also included six ST1-MSSA-t127 isolates, all of which harboured SCC*fus*$_{476}$ and half (3/6) of which were PVL-positive. Clade C included one ST1-MRSA-IVa-t127 and two ST1-MSSA-t127 isolates, all three of which were PVL-negative and harboured SCC*fus*$_{476}$. The clade C MRSA isolate was relatively closely related (38 cgSNVs) to one of the MSSA isolates, while the remaining clade C MSSA isolate differed from its closest neighbour by 119 cgSNVs. Clade D included two PVL- and SCC-negative ST1-MSSA-t127 isolates which were indistinguishable using cgSNV analysis.

4.4 Discussion

This study confirmed the existence of a previously undefined PVL-negative CC1-MRSA-IV clone that may have emerged in South-Eastern Europe and which has become prevalent in both Ireland and Bavaria, Germany. This clone is distinctly different from MRSA matching the description of the well-defined PVL-negative CC1-MRSA-IV clone known as WA MRSA-1 (and closely related WA MRSA-57 and WA MRSA-45). Although indistinguishable using a combination of MLST, SCC*mec* typing and *spa* typing, the application of cgSNV and SCC*mec* analysis revealed a clear distinction between these clones.

Phylogenetic analysis of PVL-negative CC1-MRSA-IV from Ireland, Germany, Romania and the UAE identified two major clades (A and B1; Fig. 4.3). Clade-specific SCC*mec* characteristics in the *ccr* genes and *orfX* region indicated that the clones represented by these clades evolved from MSSA following separate SCC*mec* acquisition events. The ~4.7 kb insertion detected in the *orfX* region of the clade A SCC*mec* element would likely serve as an appropriate target region for which primers could be designed to identify the clade A clone among other CC1-MRSA-IV. Furthermore, according to the recently proposed SCC*mec* subtyping scheme (Monecke et al., 2016b), the SCC*mec* element identified in clade A constitutes a novel subtype. While this element is similar to MW2-like SCC*mec*, the former is differentiated from the latter by the absence of *dcs* and the presence of a novel SCC*mec* terminus (SCC terminus 15; Fig. 4.4). As well as serving as a potential marker gene for typing purposes, SCC terminus 15 could also be targeted during future investigations which aim to design a comprehensive set of SCC*mec* integration site-spanning primers.

Genotypic evidence strongly suggests that subclade B1 corresponds to the WA MRSA-1 clone. Indeed, the subclade B1 isolates generally harboured *blaZ*, *sak*, *scn*, *sea*, *seh*, *sek* and *seq* (Table 4.4), all of which are associated with WA MRSA-1 (Coombs et al., 2011b). Interestingly, the subclade B1 isolates which harboured SCC_{476} (which is also often identified in WA MRSA-1) were interspersed between the SCC_{476}-negative subclade B1 isolates in a cgSNV-based NJT (Fig. 4.3). This brings into question the stability of SCC_{476} and thus, the weight it is afforded while discriminating between WA MRSA-1/45/57. However, considerably more investigation into this matter is required. In contrast to subclade B1, the clade A isolates exhibited genotypic characteristics which differed comprehensively from those associated with WA MRSA-1. Specifically, the clade A

isolates generally harboured *aphA3*, *aadE*, *sat* and *tet*(K), none of which are associated with WA MRSA-1, and lacked *sea*, *sek* and *seq*, all of which are associated with WA MRSA-1 (Table 4.4) (Coombs et al., 2011b).

A 2014 study using DNA microarray profiling identified PVL-negative CC1-MRSA-IV as the predominant MRSA clonal group in a Romanian hospital between 2008 and 2012 (Monecke et al., 2014). Distinct genotypic differences were noted between this CC1-MRSA-IV clone and WA MRSA-1/45/57, and it was hypothesised that the highly prevalent CC1-MRSA-IV clone may have emerged locally due to its exhibiting marked genotypic similarities to CC1-MSSA from the same region (Monecke et al., 2014). Significantly, the Romanian isolates investigated in the present study constitute a subset of those included in the 2014 study and thus, the results of the present study strongly support the conclusions of the 2014 Romanian study. Firstly, the existence of a PVL-negative CC1-MRSA-IV clone in Romania that is distinct from WA MRSA-1/45/57 was confirmed. Secondly, the close relatedness of Romanian CC1-MRSA-IV and CC1 MSSA from the same region (which differed by as few as 47 cgSNVs) was verified, suggesting that the MRSA clone may indeed have emerged in Romania (Fig. 4.3). This possibility is further supported by the facts that (i) CC1 MSSA are common in Iași, Romania (Monecke et al., 2014), (ii) the CC1-MRSA-IV clone was predominant in Iași by 2008, at which time it had been detected in only two patients in Ireland (Table 4.2) (Monecke et al., 2014) and (iii) none of the Irish CC1-MSSA isolates investigated grouped in close proximity to clade A. However, the lack of published studies on MRSA and/or MSSA from neighbouring countries means the possibility of this clone having originated elsewhere in South-Eastern Europe cannot currently be ruled out. Alternatively, it was discussed in the 2014 Romanian study that the MSSA could also be derivatives of CC1-MRSA-IV resulting from spontaneous SCC*mec* deletions (Monecke et al., 2014). In the present study, however, the cgSNV-based NJT in Figure 4.4 shows that the Romanian CC1-MSSA isolates grouped at the base of clade A, indicating that the MRSA likely evolved from the MSSA, rather than the MSSA deriving from the MRSA by loss of SCC*mec*.

Following its emergence in south-eastern Europe, putatively in Romania, the European CC1-MRSA-IV clone spread to Ireland (Fig. 4.3; Table 4.3). While this clone was present in the Irish community by 2007, it was not detected in the Irish healthcare system until 2012 and did not become prevalent in Ireland until 2013 (Table 4.2). These findings support the suggestion outlined in Chapter 3, that the consideration of risk-factors relating

to the acquisition of MRSA in the community (and not only in healthcare settings) may be appropriate during targeted MRSA screening in Irish hospitals. Interestingly, the Irish clade A isolates (which were recovered between 2007-2017) exhibited a relatively high level of genotypic diversity (1-102 cgSNVs), suggesting that the European CC1-MRSA-IV clone may have been introduced into Ireland on several occasions. Indeed, the first two European CC1-MRSA-IV isolates identified in Ireland were recovered just 26 days apart (Table 4.2) and differed by 52 cgSNVs. Correspondingly, the rate of migration from Romania to Ireland rose dramatically in 2007 and was consistently high until 2017 (Irish CSO, 2009, 2017). Phylogenetic analysis also indicated that the European CC1-MRSA-IV clone spread to Germany (Fig. 4.3). Although only sporadically encountered in Dresden, this clone became prevalent in the German city of Regensburg between 2016-2018, where it was identified in the community and two different hospitals (Table 4.2). Remarkably, considering their recovery during a period of approximately two years, the German clade A isolates exhibited a particularly high level of genotypic diversity (12-115 cgSNVs), indicating that the European CC1-MRSA-IV clone may have been introduced into Germany on multiple occasions. In 2015, Romanians represented the second largest group (213,000 people) of foreign nationals living in Germany (Federal Office for Migration and Refugees, Germany, 2016), with a particularly high proportion residing in the Regensburg region (personal communication; Dr. Wulf Schneider, University Hospital Regensburg).

It is highly likely that the CC1-MRSA-IV clone defined in this study is present in other European countries in addition to Romania, Ireland and Germany. Indeed, PVL-negative ST1-MRSA-IV-t127 have been recovered from humans, cattle, pigs, cow's milk and goat's milk in Italy, and from rooks in Austria (Alba et al., 2015; Basanisi et al., 2017; Cortimiglia et al., 2015; Loncaric et al., 2013; Monaco et al., 2013; Normanno et al., 2015). Furthermore, where detailed, the genotypic characteristics of these PVL-negative ST1-MRSA-IV correspond to those associated with the European CC1-MRSA-IV clone defined in the present study. For example, a 2015 study reported the isolation of PVL-negative ST1-MRSA-IV-t127 from dairy cows, humans and pigs in Italy, between 2009 and 2011, which generally harboured *aphA3*, *blaZ*, *sat* and *tet*(K), while exhibiting variable *erm*(C), *sak* and *scn* carriage (Alba et al., 2015). Indeed, 19% of the clade A isolates recovered during the present study lacked genes involved in human-specific innate immune evasion and harboured a non-disrupted *hlb* gene, indicating that this clone may also be prevalent in animals (Table 4.4). Future WGS-based phylogeny studies should investigate the relatedness of these animal isolates both to each other and to the European

CC1-MRSA-IV clone defined in the present study. Furthermore, a recent Italian study (which included one of the Irish clade A isolates investigated in the present study) confirmed the presence of the European CC1-MRSA-IV clone in a paediatric hospital in Florence (Manara et al., 2018). In line with the findings of the present investigation, this Italian study estimated that the novel CC1-MRSA-IV clone diverged 6-28 years ago.

The present study has demonstrated the existence of a European PVL-negative CC1-MRSA-IV clone that is distinctly different from MRSA fitting the description of the well-characterised WA MRSA-1 clone. Furthermore, cgSNV analysis revealed that this MDR clone may have originated in Romania, before spreading to both Ireland and Bavaria, Germany. To encourage the accurate identification of this clone, a representative European CC1-MRSA-IV sequence was uploaded onto a public repository and potentially suitable marker sequences were identified to assist groups without access to WGS facilities. This study also identified a novel SCC*mec* terminus which is relevant to both the recently defined SCC*mec* subtyping system and the design of SCC*mec* integration site-spanning primers.

Chapter 5

Intra-hospital, inter-hospital and intercontinental spread of ST78-MRSA-IV from two neonatal intensive care unit outbreaks

5.1 Introduction

The immaturity of their immune systems make neonates particularly susceptible to serious infection and importantly, colonisation with MRSA increases their risk of infection (Giuffrè et al., 2015; Maraqa et al., 2011). Very low birth weight infants (i.e. those under 1.5 kg) and infants with underlying diseases and/or indwelling medical devices are most vulnerable to invasive infection (Carey et al., 2008; Giuffrè et al., 2013). Hospitalised neonates are generally cared for in NICUs. In Ireland, neonates are screened for MRSA upon admission into a NICU and weekly, thereafter (Irish Department of Health, 2013). Notably, while the high frequency of direct contact between neonates and HCWs has been implicated in the spread of MRSA in NICUs (Maraqa et al., 2011), HCW screening generally occurs in Irish NICUs only upon the identification of an infection cluster among patients, as detailed in Chapter 1, section 1.9.4 (Irish Department of Health, 2013).

While the CA CC88 clones ST78-MRSA-IV and ST88-MRSA-IV/V have achieved endemic status in particular geographic regions (Monecke et al., 2011), CC88 MRSA is not usually associated with Ireland. ST78-MRSA-IV is a PVL-negative clone that usually harbours the antimicrobial resistance genes *blaZ* and *erm*(A) (Monecke et al., 2011). First isolated in remote Western Australia in 1995 (O'Brien et al., 2009), ST78-MRSA-IV was the fourth most prevalent clone recovered in Australia in 2012, accounting for 3.6% of all MRSA detected and 5.1% of MRSA detected in the community (Coombs et al., 2014). Although a single ST78-MRSA-IV isolate has been identified in Germany (Monecke et al., 2011), reports of this clone outside of Australia are lacking. ST88 MRSA includes both PVL-positive and PVL-negative strains, often harbours the exfoliative toxin gene, *etA*, and is generally associated with SCC*mec* types IV and V, although ST88-MRSA-VI have been identified sporadically in Western Australia (Monecke et al., 2011). While prevalent in the Far East (Ozaki et al., 2009; Qiao et al., 2014; Zhang et al., 2009) and predominant in Africa, where it accounts for 24.2-83.3% of all MRSA isolates (Breurec et al., 2011; Schaumburg et al., 2011), ST88 MRSA is sporadic in Europe and the Middle East (Monecke et al., 2012; Růžičková et al., 2012; Vindel et al., 2014). CC88 MRSA have also been described in bulk tank cow's milk and retail food in Saudi Arabia (Parisi et al., 2016; Raji et al., 2016). ST78 and ST88 are SLVs which differ by one nucleotide in the *tpi* locus. They are associated with *spa* types t186 and t786, the latter of which is distinguishable from the former by the absence of one repeat unit.

A suspected protracted outbreak occurred in the NICU of an Irish hospital (H1) between 2009 and 2011, in which seven CC88-associated MRSA-t186 isolates were identified during patient screening. A second suspected protracted outbreak involving a CC88-associated *spa* type occurred in the same NICU between 2014 and 2017, in which 15 MRSA-t786 screening isolates were recovered. The work described in this chapter investigated the CC88 isolates from these outbreaks with three main objectives: (1) To determine the relationship between isolates from both NICU outbreaks and to identify putative transmission events. (2) To investigate the relatedness of the NICU outbreak isolates to other CC88 MRSA identified in Ireland. (3) To investigate the relatedness of all CC88 MRSA identified at the Irish NMRSARL to international comparator isolates.

5.2 Materials and Methods

5.2.1 Isolates

All 28 CC88-MRSA isolates identified at the Irish NMRSARL between January 2009 and February 2017 were investigated. Twenty-two of these isolates were recovered in the NICU of H1 during two suspected outbreaks, which occurred between 2009 and 2011 (seven isolates from seven inpatients), and 2014 and 2017 (13 isolates from 13 inpatients and two isolates from one HCW). In each case, the suspected outbreak was initially recognised due to the number of isolates identified and their recovery in a single unit of the hospital concerned (H1). While only one isolate per patient was included, two isolates were included from a single HCW, as they were recovered two years apart (in 2015 and 2017). The remaining six Irish isolates investigated constituted all other CC88-MRSA isolates identified at the Irish MRSARL during the study period i.e. between 2009 and 2017. Fifteen international CC88 MRSA isolates were also included for comparison to the Irish isolates. These comparator isolates were selected from the global *S. aureus* strain collection described in Chapter 2, section 2.6.6, based on their similarity (n = 13) or dissimilarity (n = 2) to the Irish isolates, as determined by genotyping and SCC*mec* subtyping using *in silico* DNA microarray profiling (see sections 5.2.6 and 5.2.7 below). Isolates were identified as *S. aureus* and MRSA as described in Chapter 2, sections 2.3.2 and 2.3.3, respectively. Isolates were stored as described in Chapter 2, section 2.2.1.

5.2.2 Phenotypic susceptibility testing

The susceptibility profile of each isolate was determined as described in Chapter 2, section 2.3.4.

5.2.3 *spa* typing

All 43 isolates underwent *spa* typing as described in Chapter 2, section 2.4.3.

5.2.4 WGS

All isolates underwent WGS as described in Chapter 2, section 2.5 (Table 5.1).

5.2.5 Whole genome analysis

5.2.5.1 de novo assembly

Genomes were assembled as described in Chapter 2, section 2.6.2.

Table 5.1 Whole-genome sequencing quality assurance data

Quality parameter	Average result
Trimmed read quality	36.9
N50[a]	567101.7
No. of contigs per isolate	19.5
Assembly coverage	181.5
% core-genome loci present[b]	99.7

[a]The minimum contig length required to cover half the genome.
[b]According to the previously defined core-genome multilocus sequence typing scheme (Leopold et al., 2014).

5.2.5.2 MLST

All isolate contig sets underwent traditional MLST using Ridom SeqSphere+ version 4.1 (Ridom GmbH), as described in Chapter 2, section 2.6.3.

5.2.5.3 wgMLST

Whole-genome sequencing data were analysed using the wgMLST scheme available in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium), as described in Chapter 2, section 2.6.8.

5.2.5.4 SNV analysis

Isolates confirmed to be closely related following wgMLST analysis subsequently underwent SNV analysis using a study-specific reference sequence. Isolate P6 was chosen as the reference sequence due to both its central position in the wgMLST-based MST cluster and the high quality of its assembly. SNV analysis was performed as described in Chapter 2, section 2.6.7. SNVs were called exclusively in positions shared by all samples. Only SNVs with at least 40x coverage were considered. Potentially indel-related SNVs, occurring within 12 bp of each other, were removed. Positions with ambiguous base calls and SNVs in repetitive regions were excluded.

5.2.5.5 MST generation

MSTs were constructed firstly, involving the Irish isolates exclusively and secondly, involving both the Irish and international isolates. For the Irish isolates, in order to identify the most appropriate analysis method, three separate MSTs were generated based on cgMLST, wgMLST or SNV data, and were examined in tandem with all available epidemiological and genotypic information. As the Irish and international isolates were recovered over 16 years and from disparate geographic regions, the construction of a cgMLST-based MST was deemed appropriate in this instance. All MSTs were generated as described in Chapter 2, section 2.6.10.

5.2.6 Genotyping and SCC*mec* subtyping using *in silico* DNA microarray profiling

All Irish isolates underwent genotyping and SCC*mec* subtyping using *in silico* DNA microarray profiling, as described in Chapter 2, section 2.6.5.

5.2.7 Selection of international isolates

The 15 international isolates investigated were selected from the extensive global database described in Chapter 2, section 2.6.6. Thirteen international CC88-MRSA isolates with similar genotypic characteristics (i.e. general genotypic profile and SCC*mec* subtype) to any of the Irish isolates were selected for comparison purposes. Two international CC88-MRSA isolates with dissimilar genotypic characteristics to all of the Irish isolates were selected as controls.

5.2.8 Sequence-based plasmid analysis

Sequence-based plasmid analysis was performed at Abbott (Alere Technologies, GmbH). All sequence read sets were assembled using SPAdes v3.11.1 (Bankevich et al., 2012) with a final kmer size of 127. All contigs under 500 bp and all contigs with kmer coverage less than 3.0 were excluded. For each isolate, a scatter plot was generated depicting the GC content versus coverage for each contig. Putative plasmid-derived contigs were differentiated from chromosomal-derived contigs based on their elevated coverage, low GC content and the identity of their first and last 127 nucleotides, indicative of a circular replicon. All putative plasmid-derived contigs were blasted against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and those which mapped to a known plasmid sequence in GenBank were considered to be confirmed plasmids. Any plasmid types present in the Irish isolates or in both the Irish and international isolates were identified. For each such plasmid type, a multi-sequence global alignment was constructed including all the newly identified plasmid sequences and the GenBank reference sequence, using MAFT v7.273 (Katoh et al., 2002).

5.3 Results

5.3.1 MRSA isolates

The epidemiological, phenotypic and genotypic data associated with the 43 isolates investigated are detailed in Table 5.2. The six non-H1 CC88-MRSA isolates from Ireland were recovered in the NICU of a second Irish hospital (H2; *n* = 3) and in three additional Irish hospitals (H3, *n* = 1; H4, *n* = 1; H5, *n* = 1). All Irish isolates were recovered from colonisation sites. Each isolate is represented by a letter, indicating recovery from either a patient (P) or healthcare worker (W), followed by a number, indicating the order in which the isolates were recovered (Table 5.2). The 13 international CC88-MRSA isolates exhibiting similar DNA microarray profiles to any of the Irish isolates were recovered between 2001 and 2017 in Australia (*n* = 4), France (*n* = 3), Germany (*n* = 3), Tanzania (*n* = 2) and Egypt (*n* = 1; Table 5.2). Each of these isolates is represented by a letter, indicating the country of recovery, followed by a number, indicating the order in which the isolates were recovered (Table 5.2). The two international CC88-MRSA isolates exhibiting dissimilar DNA microarray profiles to all of the Irish isolates were recovered in Germany and are represented by the letter R, followed by a number (Table 5.2).

5.3.2 Two distinct clusters of CC88-MRSA isolates

The majority of the 28 Irish isolates investigated were identified as *spa* type t786 (*n* = 21), while those remaining were identified as t186 (*n* = 7; Table 5.2). All seven t186 isolates were recovered during the first suspected H1 outbreak, while the t786 isolates were recovered either during the second suspected H1 outbreak (*n* = 13) or from four additional hospitals (H2-H5; Table 5.2). The majority of Irish isolates (25/28) were also identified as ST78-MRSA-IVa, harbouring an SCC*mec* type IVa element corresponding to that identified in the MW2 MRSA strain (GenBank accession: BA000033.2). This included all t186 isolates (7/7) and 18/21 t786 isolates. The remaining three t786 isolates were assigned to ST88, a SLV of ST78, and harboured a SCC*mec* type IVa element corresponding to that identified in the CMFT503 MRSA strain (GenBank accession: HF569113.1; Table 5.2). The presence of a hypothetical SCC*mec* terminus protein, Q9XB68, in the MW2-like SCC*mec* element and the presence of both an alternate SCC*mec* terminus, SCC*mec* terminus 01, and the LytTR domain DNA-binding regulator, Q931B7, in the CMFT503-like SCC*mec* element, distinguished the two SCC*mec* type IVa elements (Monecke et al., 2016).

Table 5.2 Epidemiological, phenotypic and genotypic details of the 43 CC88-MRSA isolates recovered in Ireland, Australia, Egypt, France, Germany and Tanzania between 2001 and 2017

Source	Isolate/ patient no.	Month & year of recovery	Sequence type[a]	SCC*mec* type/ subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Ireland-H1	P1	Jan 2009	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P2	Apr 2009	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P3	Mar 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P4[f]	Nov 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P5[f]	Nov 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P6	Aug 2011	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P7	Sep 2011	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, sak, scn, sec, sel*
	P10	Mar 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P11	Mar 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*

Table 5.2 continued overleaf

Source	Isolate/ patient no.	Month & year of recovery	Sequence type[a]	SCC*mec* type/ subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Ireland-H1	P13	Sep 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P14	Oct 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P15	Nov 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P18	Jan 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm(A), mecA, lukX, sak, scn, sec, sel*
	W19[g]	Jan 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P20	June 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P21	Sep 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P22	Oct 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P23	Nov 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P24	Feb 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*

Table 5.2 continued overleaf

Source	Isolate/ patient no.	Month & year of recovery	Sequence type[a]	SCC*mec* type/ subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Ireland-H1	P26	June 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
	P27	June 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
	W28[g]	Feb 2017	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
Ireland-H2	P8	Aug 2013	88	IVa- CMFT503	t786	Ap, Cm, Fx, Tp	*blaZ, cadX, cat, dfrA*, *mecA*, *hsdS, lukX, sak, chp, scn*
	P16	Nov 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
	P17	Dec 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
Ireland-H3	P12[h]	Sep 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, lukX, sak, scn, sec, sel*
Ireland-H4	P9	Sep 2013	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, etA*, *hsdS, lukX, sak, chp, scn*
Ireland-H5	P25	Mar 2016	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, etA*, *hsdS, lukX*, *sak, chp, scn*
Australia	A1	2001	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, hsdS, lukX, sak, scn*

Table 5.2 continued overleaf

Source	Isolate/ patient no.	Month & year of recovery	Sequence type[a]	SCC*mec* type/ subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Australia	A2	2002	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, hsdS, lukX, sak, scn, sec, sel*
	A3	2008	78	IVa-MW2	t186	Ap, Er, Fx, Tp	*blaZ, cadX, erm*(A)*, mecA, hsdS, lukX, sak, scn, sec, sel*
	A4	2008	78	IVa-MW2	t186	Ap, Fx, Tp	*blaZ, cadX, erm*(A)*, mecA, hsdS, lukX, sak, scn, sec, sel*
Egypt	E1[i]	2014	88	IVa- CMFT503	t13712	Ap, Fx, Tp	*blaZ cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*
France	F1	2002	88	IVa- CMFT503	t186	Ap, Er, Fx, Tp	*blaZ, cadX, dfrS1*, *erm*(C), *mecA, etA*, *hsdS, lukX, sak, chp, scn*
	F2	2002	88	IVa- CMFT503	t786	Ap, Er, Fx, Tp	*blaZ, cadX, dfrS1*, *erm*(C), *mecA, etA*, *hsdS, lukX, sak, chp, scn*
	F3	2002	88	IVa- CMFT503	t690	Ap, Fx, Te, Tp	*blaZ, cadX, dfrS1*, *mecA, tet*(K), *vga*(A), *etA*, *hsdS, lukX, sak, chp, scn*
Germany	G1	2008	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A)*, mecA, hsdS, lukX, sak, scn*
	G2	2016	88	IVa- CMFT503	t1028	Ap, Fx, Te, Tp	*blaZ, cadX, dfrS1*, *mecA, tet*(K), *hsdS, lukX, sak, chp, scn*
	G3	2017	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*

Table 5.2 continued overleaf

Source	Isolate/ patient no.	Month & year of recovery	Sequence type[a]	SCC*mec* type/ subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Germany	R1	2014	88	IVa-MW2	t17863	Ap, Er, Fx	*blaZ, cadX, erm*(C), *mecA, hsdS, lukF, lukS, lukX, sak, chp, scn*
Tanzania	R2	2017	88	IVa-MW2	t5041	Ap, Fx, Te, Cp	*blaZ, cadX, mecA, tet*(K), *hsdS, lukF, lukS, lukX, sak, chp, scn*
	T1	2016	88	IVa- CMFT503	t690	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*
	T2	2016	88	IVa- CMFT503	t690	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, vga*(A), *etA*, *hsdS, lukX, sak, chp, scn*

[a]Sequence types (STs) were assigned using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany). Allelic profiles: ST78, 22-1-14-23-12-53-31; ST88, 22-1-14-23-12-4-31.

[b]All SCC*mec* subtypes were detected using either real-life or *in silico* DNA microarrays (Monecke *et al.*, 2016). Both SCC*mec* subtypes IVa-MW2 (GenBank accession: BA000033.2) and IVa-CMFT503 (GenBank accession: HF569113.1) have been described previously (Monecke *et al.*, 2016).

[c]All H1 t186 isolates were involved in an outbreak between 2009 and 2011. All H1 t786 isolates were involved in an outbreak between 2014 and 2017. *spa* repeat successions: t186, 07-12-21-17-13-13-34-34-33-34; t786, 07-12-21-17-13-34-34-33-34; t690, 07-12-21-17-13-13-34-34-34-33-34; t1028, 07-34-33-34; t5041, 07-12-21-17-13-13-34-34-34-34-33-34; t13712, 07-12-21-17-13; t17863, 07-12-12-13-13-13-34-33-34.

[d]Antimicrobial resistance phenotypes were determined by testing the susceptibility of isolates to a panel of 21 antimicrobial agents including amikacin, ampicillin (Ap), cefoxitin (Fx), chloramphenicol (Cm), ciprofloxacin (Cp), clindamycin, erythromycin (Er), fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, neomycin, rifampicin, spectinomycin, streptomycin, sulphonamide, tetracycline, tobramycin, trimethoprim (Tp) and vancomycin.

[e]All antimicrobial resistance and virulence-associated genes were detected using either real-life or *in silico* versions of the *S. aureus* Genotyping Kit 2.0 system (Abbott [Alere Technologies GmbH], Jena, Germany).

[f]Isolates P4 and P5 were recovered from twins on the same day.

[g]Isolates W19 and W28 were recovered from the same healthcare worker two years apart.

[h]The patient from whom isolate P12 was recovered had been transferred from H1.

[i]Isolate E1 was recovered from a buffalo. All other isolates were recovered from humans.

Abbreviations: H, hospital; NA, not available

Interestingly, a highly conserved gene within *S. aureus* lineages, the type I restriction modification system gene, *hsdS* (Waldron and Lindsay, 2006), was absent from all t786 ST78-MRSA-IVa isolates. It was subsequently noted that, according to the cgMLST-based MST, this unusual deletion had occurred twice within a relatively small population (Fig. 5.1). Importantly, however, the wgMLST-based MST (Fig. 5.2) indicated that the *hsdS* deletion occurred just once within this population. It was therefore concluded that the wgMLST-based tree likely depicted the evolutionary path of this strain more accurately than the cgMLST tree. To confirm/dispute this finding, a SNV-based MST was generated involving the relevant isolates (Fig. 5.3). The structure of this tree was in agreement with that of the wgMLST MST, confirming that the *hsdS* deletion likely occurred once during the strain's spread. Ultimately, considering that the application of SNV analysis was not appropriate for all 28 Irish isolates, the wgMLST-based MST was selected for detailed data interpretation. Therefore, any allelic distances stated herein between Irish isolates exclusively, refer to wgMLST loci, while those stated between Irish and international isolates, or between international isolates exclusively, refer to cgMLST loci, as outlined in the methods.

Whole-genome MLST further supported the differentiation of isolates as suggested by their SCC*mec* subtypes and traditional STs, grouping all 25 ST78-MRSA-IVa isolates into a large cluster at one end of a MST, while the three ST88-MRSA-IVa isolates dispersed at the opposite end of the tree. The ST78-MRSA-IVa and ST88-MRSA-IVa isolates differed by a minimum of 325 alleles and exhibited average pairwise allelic distances of 23.8 (min. = 1; max. = 71) and 140.7 (min. = 81; max. = 211), respectively (Fig. 5.2).

5.3.3 ST78-MRSA-IV

The 25 ST78-MRSA-IVa isolates which grouped into the large wgMLST-MST cluster were recovered at intervals of 0-30 months and included all H1 isolates, two H2 isolates (P16 and P17) and the H3 isolate (P12). All patients from whom ST78-MRSA-IV isolates were recovered were neonates. The patient from whom the H3 isolate was recovered, had recently been transferred from H1. All ST78-MRSA-IV isolates exhibited resistance to ampicillin and erythromycin, and harboured *blaZ*, *erm*(A), *cadX* (encoding cadmium tolerance), IEC genes *sak* and *scn* (IEC type E) and enterotoxin genes *sec* and *sel* (Table 5.2). Isolate P7 was the only isolate that lacked the leukocidin homologue, *lukX*. The maximum distance observed between any two directly linked nodes was 32 alleles, detected between t186 isolates, P1 and P7, which were recovered almost three years apart

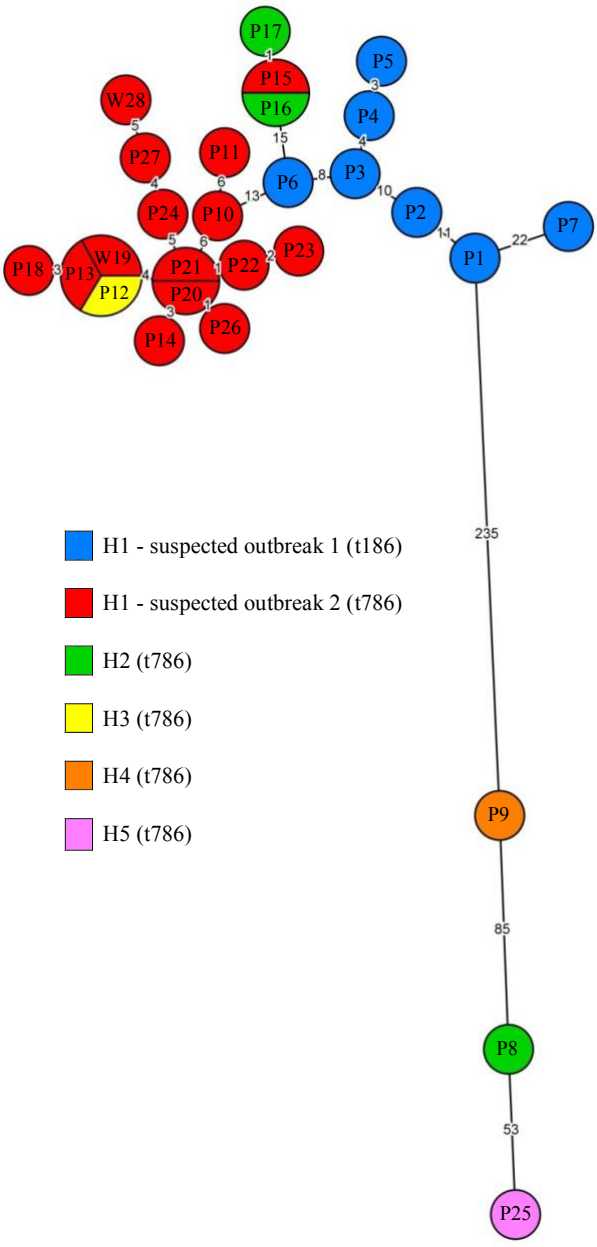


Figure 5.1 A minimum spanning tree (MST) based on cgMLST profiles of 28 CC88-MRSA isolates recovered in Irish hospitals between 2009-2017. Isolates are numbered in the order in which they were recovered. Isolate *spa* types are indicated in the colour legend. Suspected outbreak 1, involving seven t186 isolates, occurred in the NICU of H1 between 2009 and 2011. Suspected outbreak 2, involving 15 t786 isolates, occurred in the same NICU between 2014 and 2017. Branch labels represent allelic distances. All suspected outbreak 1 isolates, both H2 isolates and the H3 isolate exhibited an unusual *hsdS* deletion. The present MST suggests that this deletion occurred twice during the spread of this strain; once in a common ancestor of isolates P6 and P10 and again, in a common ancestor of isolates P6 and P15. However, both wgMLST and SNV data suggest that this deletion occurred only once, in a common ancestor of isolates P6 and P10. Abbreviations: H, hospital; NICU, neonatal intensive care unit.

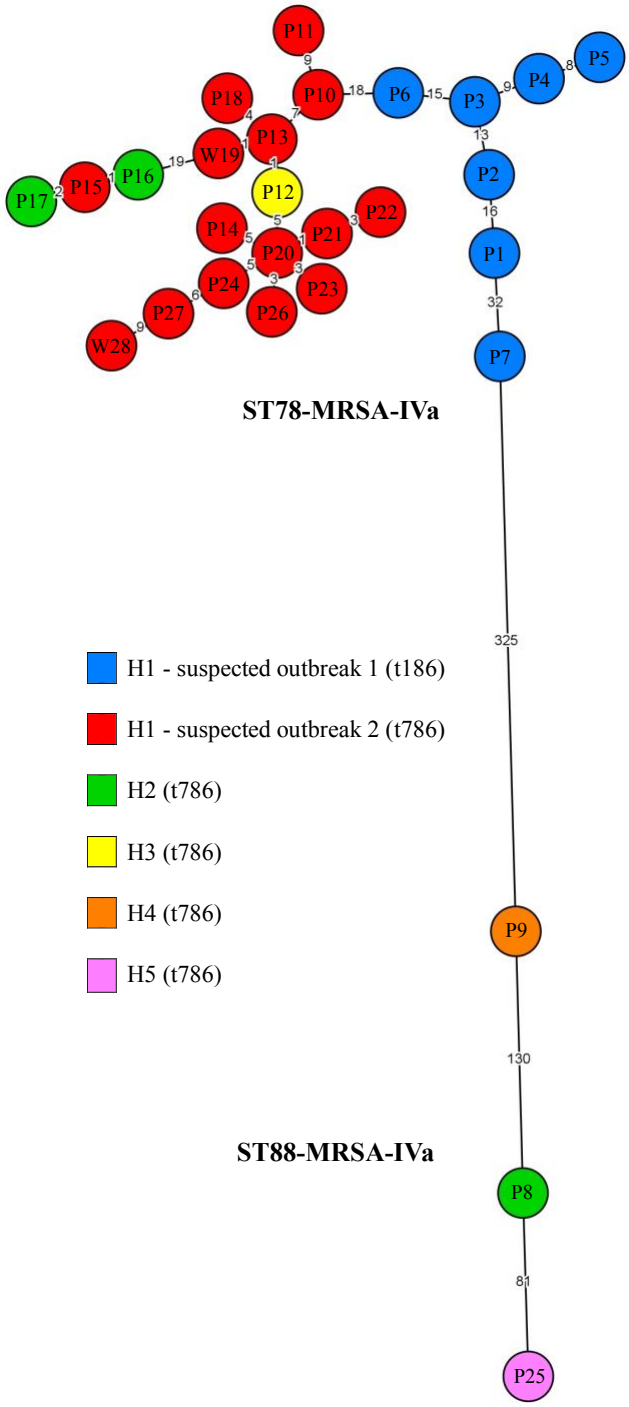


Figure 5.2 A minimum spanning tree based on wgMLST profiles of 28 CC88-MRSA isolates recovered in Irish hospitals between 2009-2017. Isolates are numbered in the order in which they were recovered. Isolate *spa* types are indicated in the colour legend. Suspected outbreak 1, involving seven t186 isolates, occurred in a NICU of H1 between 2009 and 2011. Suspected outbreak 2, involving 15 t786 isolates, occurred in the same NICU between 2014 and 2017. While one isolate was included per patient, two isolates (W19 and W28) recovered two years apart were included from a single healthcare worker. The remaining isolates were recovered from four different Irish hospitals. Branch labels represent allelic distances. Abbreviations: H, hospital; NICU, neonatal intensive care unit.

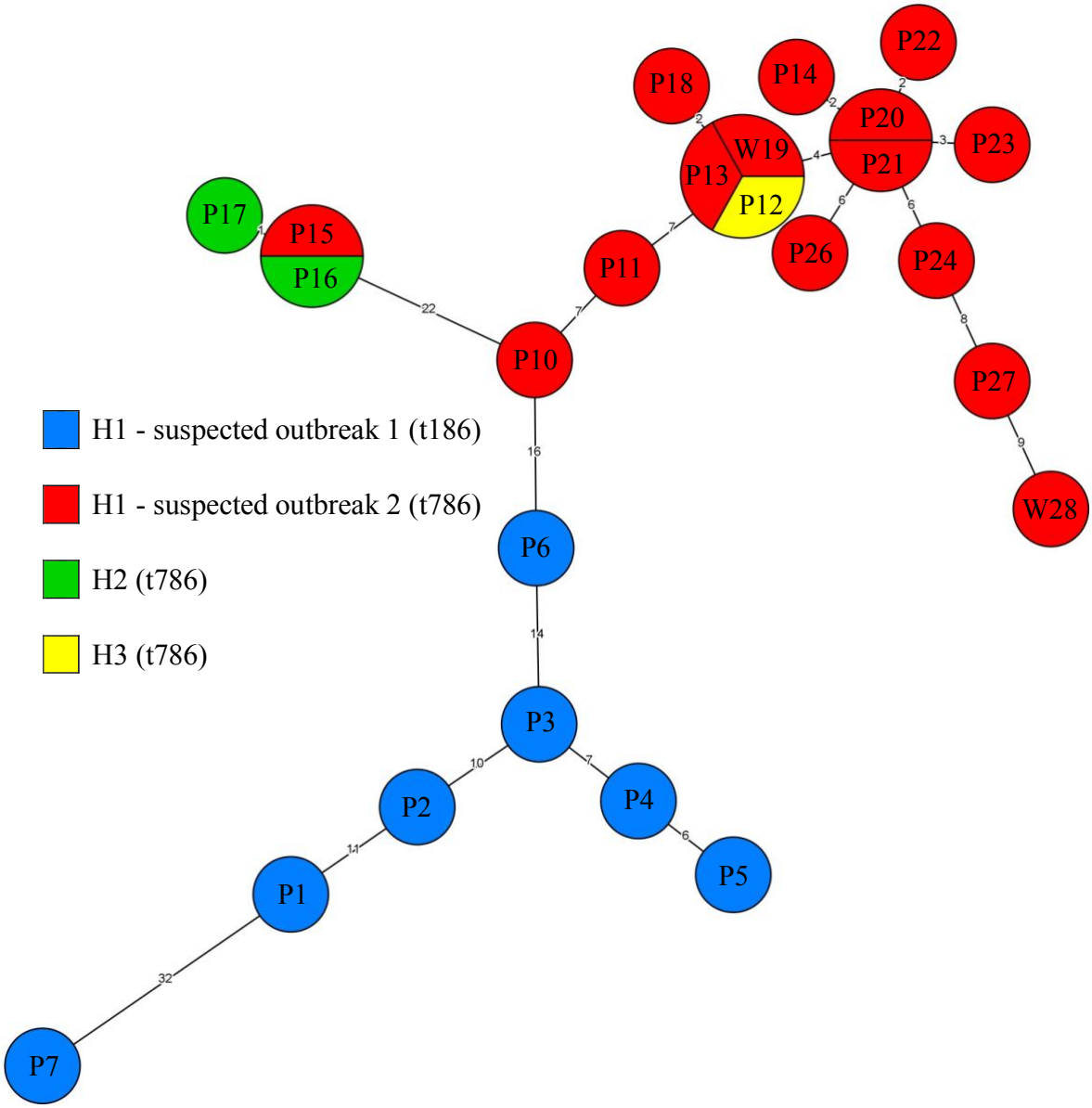


Figure 5.3 A minimum spanning tree based on a SNV analysis of 25 CC88-MRSA isolates recovered in Irish hospitals between 2009-2017. Isolates are numbered in the order in which they were recovered. Note the absence of isolates P8, P9 and P25 which were excluded from the present figure due to their distant relationship with the remaining isolates. Isolate *spa* types are indicated by the colour legend. Suspected outbreak 1, involving seven t186 isolates, occurred in a NICU of H1 between 2009 and 2011. Suspected outbreak 2, involving 15 t786 isolates, occurred in the same NICU between 2014 and 2017. Branch labels represent SNV distances. All suspected outbreak 1 isolates, both H2 isolates and the H3 isolate exhibited an unusual *hsdS* deletion. In agreement with the wgMLST-based tree shown in Figure 5.2, the present figure suggests that this deletion occurred once during the spread of this strain, in a common ancestor of isolates P6 and P10. Abbreviations: H, hospital; NICU, neonatal intensive care unit; SNV, single nucleotide variation.

during suspected outbreak 1. All other directly linked isolates exhibited 1-19 allelic differences, significantly fewer than the recently proposed approximate clonality threshold of 24 alleles (Schürch et al., 2018). This indicated a high degree of relatedness between the vast majority of directly linked isolates and a significant relationship between all isolates within the cluster network (Fig. 5.2). Furthermore, there were no apparent sub-clusters dictated by *spa* type, suggesting that the "two outbreak strains" were homogeneous. Specifically, the branch that linked isolates P6 (t186) and P10 (t786) constituted the only direct link between the t786 and t186 isolates. However, this branch represented an allelic distance of 18, lower than both those of 19 and 32, each of which was observed elsewhere in the MST cluster. Interestingly, while the largely linear structure of the t186 isolates indicated that the outbreak strain was transmitted in a relatively sequential manner between 2009 and 2011, the highly branched network of t786 isolates suggested that a more complex transmission chain was established between 2014 and 2017 (Fig. 5.2).

Isolates P4 and P5, which exhibited eight allelic differences, were recovered from twins on the same day, suggesting that parallel or sequential acquisition may have occurred in this instance (Fig. 5.2). One of the H2 isolates, P16, and a H1 isolate (P15) recovered six days before isolate P16, exhibited one allelic difference, strongly indicating that the outbreak strain spread between these two hospitals and suggesting transmission from the same source (Fig. 5.2). Isolate P16 and the second H2 isolate (P17), which was recovered 44 days after isolate P16, exhibited three allelic differences, indicating further spread of this strain in H2 (Fig. 5.2). Similarly, the only H3 isolate (P12) and a H1 isolate (P13) recovered four days after the H3 isolate, exhibited one allelic difference, clearly indicating that the outbreak strain spread from H1 to H3, and suggesting transmission from the same source (Fig. 5.2). Interestingly, the two t786 isolates (W19 and W28) recovered from the same HCW two years apart exhibited 20 allelic differences, indicating that the strain had either accumulated allelic differences over time *in vivo*, or that the HCW transiently carried different variants of the strain. Isolates W19 and W28 differed from the other t786 isolates by 1-21 (average:10.5) and 9-37 (average: 21.3) alleles, respectively, suggesting transmission of the outbreak strain between patients and the HCW (Fig. 5.2).

5.3.4 ST88-MRSA-IV

The three ST88-MRSA-IV isolates, which exhibited an average of 140.7 pairwise allelic differences, included the final H2 isolate (P8), and the H4 (P9) and H5 (P25) isolates, all of which were t786 (Table 5.2 and Fig. 5.2). None of the patients from whom ST88-MRSA-

IV isolates were recovered were neonates (patients were aged 15 months, 25 years and 66 years). Two of the ST88-MRSA isolates (P8 and P9) were recovered from patients with names suggestive of a family connection to an African country. The phenotypic resistance profiles varied slightly amongst the ST88-MRSA-IV isolates, all of which exhibited resistance to both ampicillin and trimethoprim, while isolate P8 exhibited chloramphenicol resistance (Table 5.2). The ST88-MRSA-IV isolates also exhibited slightly differing genotypic profiles, all harbouring resistance genes *dfrSI, blaZ* and *cadX*, and the IEC genes *chp*, *sak* and *scn* (IEC type B), while isolate P8 carried the chloramphenicol resistance gene, *cat*, and isolates P9 and P25 harboured *etA* (Table 5.2). Considering these differences, the lack of epidemiological links and most importantly, the number of alleles by which they differed, these three isolates did not appear to be closely related.

5.3.5 Relatedness of Irish and international CC88 MRSA

Five of the 13 international CC88-MRSA isolates exhibiting similar array profiles to the Irish isolates were identified as ST78-MRSA-IVa-MW2. This included one German isolate (G1) recovered in 2008 and four Australian isolates, A1-A4, recovered in 2001, 2002, 2008 and 2008, respectively (Table 5.2). The remaining eight international isolates exhibiting similar array profiles to the Irish isolates were identified as ST88-MRSA-IVa-CMFT503. This included three French isolates (F1-3) recovered in 2002, two Tanzanian isolates (T1 and T2) recovered in 2016, one Egyptian isolate recovered in 2014 (E1) and two German isolates, G4 and G5, recovered in 2016 and 2017, respectively (Table 5.2). The two international reference isolates (R1 and R2 recovered in Germany in 2014 and 2017, respectively) which exhibited dissimilar array profiles to the Irish isolates, were identified as ST88-MRSA-IVa-MW2. Following the construction of a cgMLST-based MST including all Irish and international isolates, international isolates R1 and R2 were excluded from further analysis as they failed to cluster with any other isolates (Fig. 5.4), differing from their (shared) most closely related isolate (A1) by 189 and 198 alleles, respectively.

5.3.6 Irish and international ST78-MRSA-IVa isolates

The five international ST78-MRSA-IVa isolates harboured the same resistance and virulence-associated genes as the Irish ST78-MRSA-IVa isolates (Table 5.2). The Irish and international ST78-MRSA-IVa isolates also exhibited very similar phenotypic susceptibility profiles, with both isolate groups exhibiting ampicillin and erythromycin resistance, while two international isolates (A3 and A4) also exhibited trimethoprim

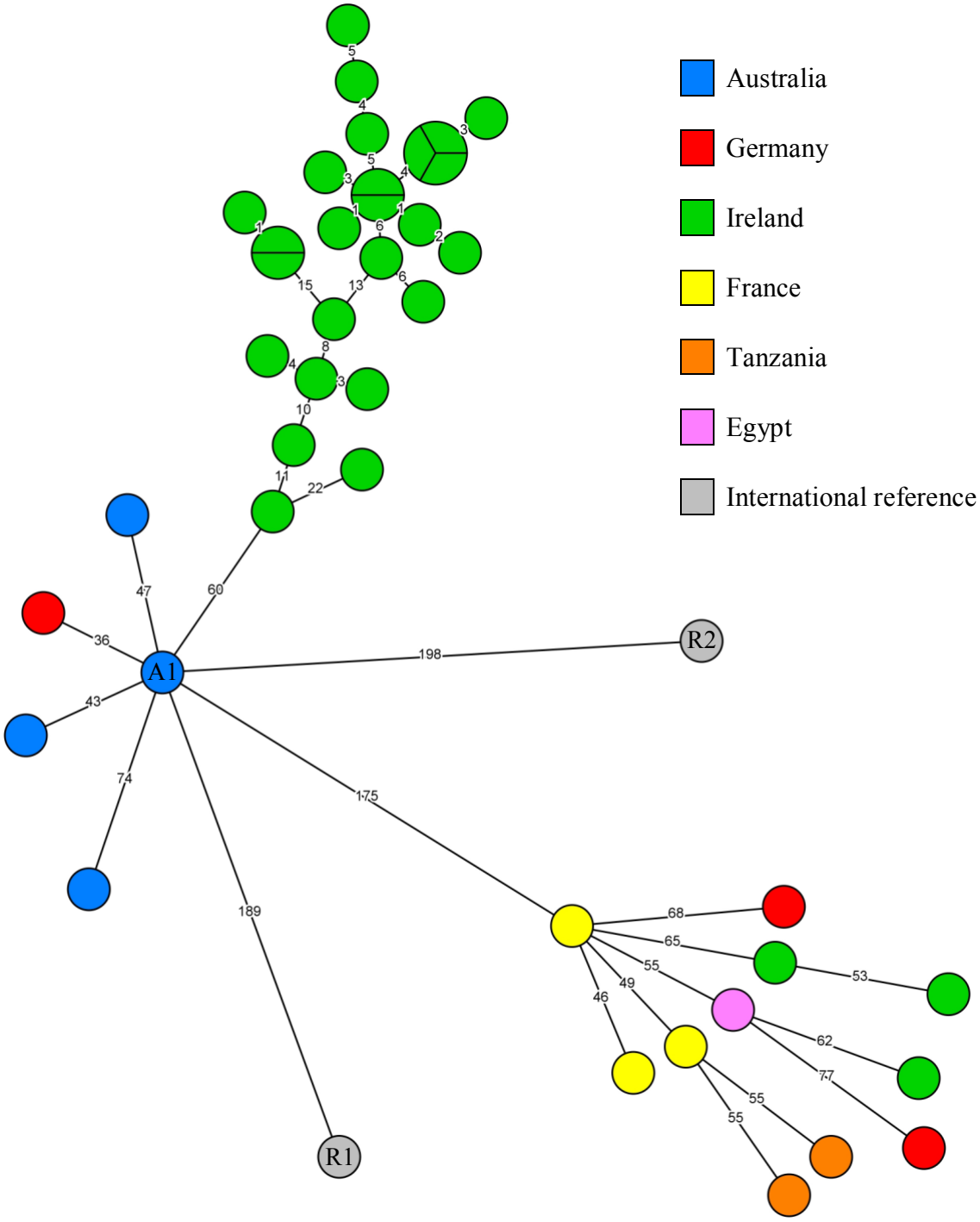


Figure 5.4 A minimum spanning tree based on cgMLST profiles of the 28 Irish and 15 international CC88-MRSA isolates investigated. Thirteen of the international isolates were selected for comparison purposes based on the similarity of their DNA microarray profiles to those of the Irish isolates. Two international isolates (R1 and R2) were selected as references based on the dissimilarity of their array profiles to those of the Irish isolates. Branch labels represent allelic distances.

resistance. Interestingly, all international ST78-MRSA-IVa isolates were identified as t186 and none exhibited the *hsdS* deletion that characterised the Irish t786 ST78-MRSA-IVa isolates. Following the generation of a cgMLST-based MST including Irish and international isolates (Fig. 5.5), the international ST78-MRSA-IVa isolates grouped in relatively close proximity to the Irish ST78-MRSA-IVa cluster and exhibited an average pairwise allelic distance of 68.7 (min. = 36; max. = 105; Fig. 5.5). Specifically, isolates A2-A4 and G1 all radiated independently from isolate A1, which was the only isolate that linked directly to the t186 side of the Irish cluster (Fig. 5.5). Isolate A1 (recovered in 2001 in Australia) and its most closely related Irish isolate (P1; recovered in 2009) exhibited 60 allelic differences which, considering the disparate geographic regions and different time periods in which they were recovered, suggested a significant degree of relatedness between these two isolates.

All Irish and international ST78-MRSA-IVa isolates harboured a 21 kb plasmid encoding *blaZ* and *cadX*, corresponding to plasmid pWBG763 (GenBank accession number: GQ900467.1). Interestingly, however, the Irish ST78-MRSA-IVa isolates were characterised by a 100 bp deletion in this plasmid. All Irish and two Australian (A2 and A3) ST78-MRSA-IVa isolates also harboured a cryptic 2 kb plasmid corresponding to pWBG764 (GenBank accession number: GQ900468.1). Both plasmids pWBG763 and pWBG764 were originally sequenced from the same ST78-MRSA-IVa strain (WBG8366), which was recovered in remote Western Australia in 1995.

5.3.7 Irish and international ST88-MRSA-IVa isolates

The eight international ST88-MRSA-IVa isolates harboured similar resistance and virulence-associated genes to the Irish ST88-MRSA-IVa, all carrying *blaZ*, *cadX*, *dfrS1* and the IEC genes *chp*, *sak* and *scn* (IEC type B), while exhibiting variable *etA* carriage (Table 5.2). However, two of the international ST88-MRSA-IVa isolates (F1 and F2) also harboured *erm*(C), while two others (F3 and G2) harboured *tet*(K), and a third pair (F3 and T2) harboured *vga*(A), none of which were present in the Irish isolates (Table 5.2). The Irish and international ST88-MRSA-IVa isolates also exhibited similar phenotypic susceptibility profiles, with both isolate groups exhibiting ampicillin and trimethoprim resistance, while two international ST88-MRSA-IVa isolates (F1 and F2) exhibited erythromycin resistance and two others (F3 and G2) exhibited tetracycline resistance, neither of which were observed in the Irish ST88-MRSA-IVa isolates. None of the international ST88-MRSA-IVa isolates exhibited chloramphenicol resistance, which was

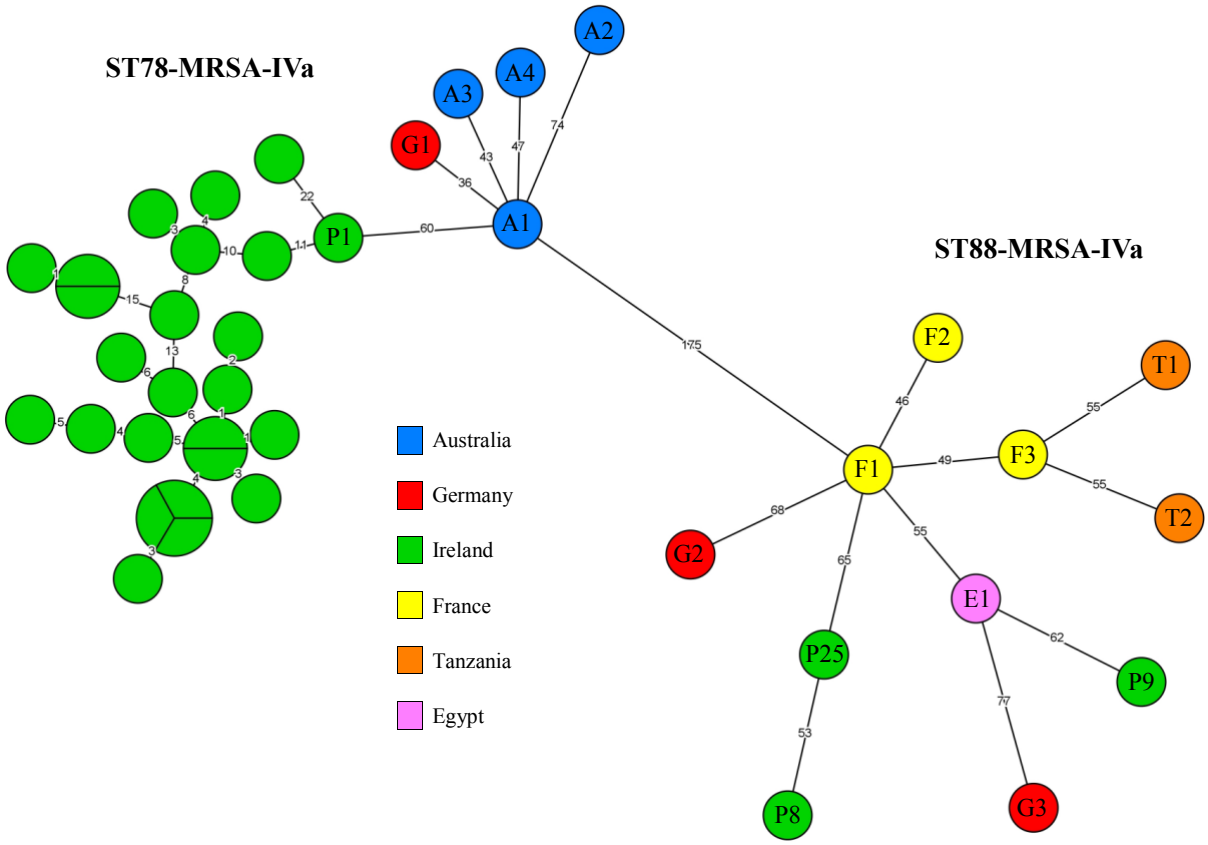


Figure 5.5 A minimum spanning tree based on cgMLST profiles of 28 Irish and 13 international CC88-MRSA isolates recovered between 2001 and 2017. The countries in which the isolates were recovered are indicated in the colour legend. Branch labels represent allelic distances. Isolates were identified as either ST78-MRSA-IVa or ST88-MRSA-IVa.

observed in one Irish ST88-MRSA-IVa isolate (P8). Isolate F1 was identified as t186, while the remaining international ST88-MRSA-IVa isolates were identified as t13712 (*n* = 1), t1786 (*n* = 2), t690 (*n* = 3) or t1028 (*n* = 1; Table 5.2). The eight international ST88-MRSA-IVa isolates formed a dispersed cluster with the three Irish ST88-MRSA-IVa isolates, in which an average pairwise allelic distance of 78.6 was observed (min. = 46; max. = 114; Fig. 5.5). Irish isolates P25 and P9 differed from their most closely related international isolates (F1 and E1, respectively) by 65 and 62 alleles, respectively, indicative of shared ancestral genotypes. Irish isolate P8 and its most closely related international isolate (F1) exhibited 188 allelic differences, suggesting a lack of relatedness between the two isolates (Fig. 5.5).

All Irish and international ST88-MRSA-IVa isolates harboured a 25 kb plasmid encoding *blaZ* and *cadX*, which corresponded to contig 10 (GenBank accession number: FMNJ01000010.1) of a previously published WGS project involving an MRSA isolate (GenBank accession number: FMNJ01000000.1) recovered in Tanzania in 2008. Irish ST88-MRSA-IVa isolate P8 also harboured a *cat*-encoding plasmid, which corresponded to contig 32 (GenBank accession number: LFNS01000032.1) of a previously published WGS project involving an ST3019 *S. aureus* isolate recovered in Ghana in 2013 (GenBank accession number: LFNS00000000.1).

5.4 Discussion

The present study revealed the homogeneity of isolates involved in two outbreaks in the NICU of an Irish hospital. Although isolate *spa* types and recovery dates suggested that two different CC88-MRSA strains may have been involved in the outbreaks in this NICU, wgMLST revealed that these outbreaks were caused by the same CC88/ST78-MRSA-IVa strain, which spread within the ward during two separate transmission periods (transmission periods 1 and 2). This investigation highlighted both the involvement of a HCW in the outbreak transmission chain and the strain's spread to two other Irish hospitals. A cgMLST-based comparison with international comparator isolates revealed that the outbreak strain was most likely imported from Australia, where it is among the prevalent MRSA clones. This study also identified a second CC88-MRSA clone present in Irish hospitals, ST88-MRSA-IVa, which was likely imported from Africa, where it is predominant, and/or a country with a large population of African ethnic origin.

Transmission period 1 (TP1) involved the intermittent acquisition of the outbreak strain by seven NICU patients over a 32-month period. Interestingly, the topology of the MST indicated that all TP1 isolates, apart from P7, were acquired in a relatively sequential chain of transmission. While the topological characteristics of a phylogenetic tree can reveal invaluable relatedness and transmission details, both previously published studies and epidemiological data must also be drawn upon in order to gain meaningful insights into the dynamics of an outbreak. Notably, previous studies have indicated that patient-to-patient transmission is rare in adult intensive care units (Price et al., 2014; Wesley Long et al., 2014), a finding which is likely applicable to NICUs given the dependency of neonates on adults for mobility. Furthermore, it is unlikely that patients P1-P6 had overlapping stays (excluding twins, P4 and P5), considering the dates on which their isolates were recovered (Table 5.2). It may therefore be concluded that patient-to-patient transmission did not play a significant role in the outbreak during this period. Similarly, given that previous research suggests that *S. aureus* can survive a maximum of 90 days on hospital plastics and fabrics (Neely and Maley, 2000), and isolates P1-P6 were recovered at intervals of 4-11 months (Table 5.2), it is perhaps unlikely that patients P1-P6 acquired the outbreak strain directly from their environment without the involvement of another intermediary factor(s). Finally, previous studies have identified a role for HCWs in the transmission of MRSA to NICU patients (Azarian et al., 2016; Brennan et al., 2012; Geva et al., 2011). Considering these points, it appears highly likely that HCWs were involved in the spread of the outbreak strain during TP1, although their exact role cannot be definitively determined. These

considerations, in combination with the topology of the MST, indicate that more than one vector was involved in spreading the outbreak strain during TP1. The data suggest two possible scenarios. Firstly, it is possible that a HCW constituted the primary outbreak source, originally seeding the outbreak strain in 2009 and transmitting it to patient P1, before seeding the strain again in 2011 and transmitting it to patient P7, while a different HCW initiated the strain's spread to patients P2-P6 (Table 5.2; Fig. 5.2). Alternatively, the data suggest that patient P1 constituted the primary outbreak source and, while one HCW initiated transmission to patients P2-P6, a different HCW, who had also acquired the strain in 2009, eventually transmitted it to patient P7.

Interestingly, the outbreak strain identified here was not detected between 2011 and 2014. It is unknown whether any staff changes or staff decolonisation occurred in H1 during this intervening period. Upon reappearing in 2014, the outbreak strain had undergone slight modifications which were detectable using conventional molecular epidemiological typing. Specifically, *spa* typing indicated that the *spa* gene had evolved from t186 to t786, while DNA microarray profiling revealed an unusual *hsdS* deletion. It is highly likely that these alterations occurred locally, either while the strain resided *in-vivo* in a H1 HCW, or during the strain's spread in the local community, prior to reintroduction into the NICU.

Transmission period 2 (TP2) involved the acquisition of the outbreak strain, ST78-MRSA-IVa, by 16 patients and one HCW, over a 35-month period. Interestingly, the MST indicated that the vector from which patient P6 acquired the outbreak strain (during TP1), may have constituted the source of the outbreak at the beginning of TP2 (Fig. 5.2). Furthermore, as observed during TP1, it appeared that more than one vector was involved in the spread of the outbreak strain during TP2. This was evident from the significant extension of three TP2 isolates (P15, P16 and P17, recovered in H1, H2 and H2, respectively) from the main body of the cluster in which isolates with both earlier and later recovery dates resided (Fig. 5.2). In contrast to TP1, however, the TP2 isolates were recovered at intervals of 0-7 months, suggesting that some TP2 patients may have had overlapping stays (Table 5.2). This circumstance may have contributed to the establishment of a more complex transmission chain during this time period. It appears that the presence of this unusual strain may have prompted HCW screening during TP2, the extent of which, is unknown. Importantly, however, an average pairwise distance of 10.5 (range: 1-21) between a H1 HCW isolate (W19) and the remaining TP2 isolates indicated that this HCW was likely directly involved in transmitting the outbreak strain to patients

during this period. Similarly, a difference of one allele between both a H1/H2 (P15 and P16) and H1/H3 (P13 and P12) isolate pair, indicated that the outbreak strain spread to two additional hospitals. In the case of H3, it is highly likely that patient P12 acquired the outbreak strain in H1, before being transferred to H3. Similarly, although no known patient transfers occurred between H1 and H2, it is possible that a carrier who was not represented in the present study (i.e. a patient not screened during routine surveillance) was transferred from H1 to H2, during TP2. This is particularly feasible given the high frequency with which patients are transferred between Irish hospitals. However, as the employment of specialist healthcare staff by different hospitals is not uncommon in Ireland, it remains possible that the movement of staff facilitated the inter-hospital spread of this strain.

Genotypic data from both the present investigation and previously published studies were considered while determining the putative geographic origin(s) of the outbreak strain. Firstly, cgMLST indicated that the outbreak strain shared an ancestral genotype with an isolate recovered in Australia (Fig. 5.5). Furthermore, a 21 kb *blaZ* and *cadX*-encoding plasmid was detected in all Australian and Irish ST78-MRSA-IVa isolates, while a second cryptic 2 kb plasmid was detected in two Australian and all Irish ST78-MRSA-IVa isolates. Moreover, both of these plasmids were previously sequenced from the same Australian ST78-MRSA-IVa strain. Finally, ST78-MRSA-IVa is generally reported exclusively from Australia and the rate of travel between Australia and Ireland was consistently high in the years preceding the study period (Australian Government Department of Immigration and Citizenship, 2011). Considering these points, it is highly likely that the outbreak strain was imported from Australia, where it is commonly known as WA MRSA-2 (Coombs et al., 2012). Interestingly, a 2012 study reported that while ST78-MRSA-IVa was the second most prevalent strain among HCWs in a Western Australian hospital, it was associated exclusively with persistent carriage (Verwer et al., 2012). This suggests, that even without constituting the predominant clone in a hospital setting, ST78-MRSA-IV colonisation may be particularly likely to persist, a phenomenon which may have contributed the continued spread of this strain to H1 NICU patients over the eight-year study period. A second CC88-MRSA clone, ST88-MRSA-IVa, was also identified in Irish hospitals during the present study. In Ireland, patients with specific HCA-MRSA risk factors generally undergo screening for MRSA (Irish Department of Health, 2013). These guidelines likely formed the basis upon which three ST88-MRSA-IVa isolates were recovered from three different patients during the study period (P8, P9 and P25). However, considering both the lack of epidemiological links between these

isolates and more importantly, the high number of alleles by which they differed, it was concluded that this strain was introduced into Irish hospitals on three separate occasions (Fig. 5.2). Moreover, the non-neonatal status of these patients further supported the likelihood of their having acquired this strain (generally considered CA) outside of a healthcare setting, prior to admission.

Extensive genotypic, conventional epidemiological and previously published data were all considered while determining the region(s) from which ST88-MRSA-IVa was likely imported into Ireland. Firstly, cgMLST indicated that two Irish ST88-MRSA-IVa isolates, P9 and P25, shared ancestral genotypes with isolates recovered in Egypt and France, respectively (Fig. 5.5). Furthermore, sequence-based plasmid analysis revealed that all Irish and international ST88-MRSA-IVa isolates harboured the same *blaZ* and *cadX*-encoding plasmid, previously sequenced from a Tanzanian MRSA isolate. This suggested that all ST88-MRSA-IVa investigated may have originated in relatively close geographic proximity. Moreover, an Irish ST88-MRSA-IVa isolate harboured an additional plasmid, previously sequenced from a Ghanaian *S. aureus* isolate. Secondly, ST88 MRSA has become increasingly associated with Africa in recent years and France is known to have a large population of African ethnic origin (Schaumburg et al., 2014; https://www.insee.fr/en/statistiques/1283070). Finally, two of the three patients from whom ST88-MRSA-IVa was recovered, had African names, suggesting they may have had family connections to an African country. Considering these points, it was concluded that ST88-MRSA-IVa was likely imported into Ireland from Africa and/or a country with a large population of African ethnic origin.

While WGS and DNA microarray profiling were successfully utilised to achieve the aims of the present study, two significant limitations, which often impede WGS-based studies, were also identified. Firstly, in the absence of universally accepted WGS data interpretation guidelines, the most probable number of hosts responsible for spreading the outbreak strain could not be definitively determined. Secondly, a lack of detailed epidemiological information limited the certainty with which conclusions could be drawn regarding the intricate dynamics of the H1 NICU outbreak, thus highlighting the importance of strong communicative links between HCFs and research groups. Finally, this study highlighted the importance of considering all available epidemiological and genotypic information while selecting the whole-genome analysis approach best suited to the specific data set in question.

The present study revealed the HCW-facilitated spread of an Australian CA-MRSA strain, ST78-MRSA-IVa, in a NICU of an Irish maternity hospital over an eight-year period. Such findings indicate that further consideration of the role of HCWs in the transmission of MRSA in high-dependency units, such as NICUs, may be beneficial. This study also identified multiple introductions of an African CA-MRSA clone, ST88-MRSA-IVa, into Irish hospitals, suggesting that CA-MRSA risk factors should perhaps be considered during targeted patient screening. In a broader context, this study highlighted both the significance of travel in the spread of MRSA and the need for well-designed studies that aim to establish WGS data interpretation guidelines.

Chapter 6

The transmission of *Staphylococcus aureus* between healthcare workers, patients and the environment, in a large Irish hospital

6.1 Introduction

An in-depth understanding of the transmission dynamics of MRSA is required in order to develop effective IPC strategies. Several major routes and modes of MRSA transmission have been acknowledged in the literature and reflected in standard IPC procedures in hospitals/HCFs. Firstly, patients have been identified as a major reservoir for MRSA, and patient screening/decolonisation is therefore common practice in many European countries (Kinoshita et al., 2017). Targeted patient screening is generally accepted as practical in MRSA-endemic regions, while universal screening is feasible in countries with low MRSA rates (Cairns et al., 2014; Vos et al., 2009). Secondly, environmental surfaces have been recognised as important MRSA reservoirs, and effective disinfection regimes therefore constitute an integral component of standard IPC policies (Creamer et al., 2014; HSE, 2005). Near-patient areas such as bedside lockers, mattresses and bed frames have previously been identified as significant sources of MRSA (Creamer et al., 2014). Thirdly, the aerial dispersion of MRSA has been identified as a mode of transmission and where possible, MRSA-positive patients are isolated or cohorted (Creamer et al., 2014; Irish Department of Health, 2013). Additionally, specialised air filtering systems may be used to remove large air-borne droplets to which pathogens such as MRSA, may be attached (Qian et al., 2010). Finally, it has been shown that HCWs can transmit MRSA to patients, and comprehensive IPC regimes therefore include varying degrees of HCW screening/decolonisation (Irish Department of Health, 2013; Vos et al., 2009). While routine HCW screening has been justifiably adopted in countries with low MRSA rates (Humphreys et al., 2009; Vos et al., 2009), robust evidence exists only to indicate that HCW screening is appropriate in MRSA-endemic regions during outbreaks (as highlighted in Chapters 3 and 5) (Brennan et al., 2012; Harris et al., 2013; Madigan et al., 2018). Interestingly, however, it has been suggested that countries with high MRSA rates may also benefit from routine HCW screening (Hawkins et al., 2011; Higgins et al., 2010). Currently, there exists little evidence to support or dispute this suggestion as few studies have investigated whether HCWs constitute a significant source of MRSA in patients in non-outbreak MRSA-endemic settings.

In addition to investigating the spread of MRSA, it is also important to increase our understanding of MSSA transmission. This necessity has been highlighted by recent European trends which demonstrate that invasive MSSA infections are becoming significantly more common (EARS-Net, 2017). Specifically, studies indicate that while the number of *S. aureus* BSIs per annum has remained consistently high over the past decade,

the proportion of MRSA BSIs has decreased and the proportion of MSSA BSIs has increased (EARS-Net, 2009, 2017). Compared to MRSA, however, tracking the spread of MSSA presents a unique set of challenges. Indeed, while the vast majority of MRSA worldwide belong to a limited number of particularly successful clonal groups, MSSA include a highly diverse range of genotypes (Grundmann et al., 2010, 2014; Miko et al., 2013). Furthermore, while MRSA clones are often associated with specific geographic regions, MSSA genotypes do not exhibit any clear temporal or geographic clustering patterns (Grundmann et al., 2010, 2014; Miko et al., 2013). Finally, MSSA lack SCC*mec* which is a useful source of marker sequences in MRSA clones. These characteristic features of MSSA further diminish the applicability of conventional molecular typing for these organisms, however, very few studies have used WGS to investigate the transmission of MSSA.

In order to help determine whether MRSA-endemic regions would benefit from the routine screening of HCWs for MRSA, the primary aim of this study was to investigate the potential role of HCWs in the transmission of *S. aureus* to patients in a non-outbreak scenario, using WGS. Furthermore, considering that MSSA are becoming increasingly associated with hospital-acquired infections, the secondary aim of this study was to thoroughly investigate the molecular epidemiology of *S. aureus* in a large Dublin hospital using a combination of WGS and phenotypic antimicrobial resistance testing.

6.2 Materials and methods

6.2.1 Setting

The present study was conducted in an 820-bed teaching hospital in Dublin, between May and October in 2017. The study hospital has approximately 25,000 admissions per year and 3,000 staff members. Bed occupancy is generally 100%. This investigation forms part of a larger Irish Health Research Board-funded study for which the Beaumont Hospital Ethics (Medical Research) Committee granted ethical approval (reference number: 17/01) on the 16th of February 2017.

6.2.2 Volunteer recruitment

Volunteers were recruited on nine different hospital wards (A-I), the details of which are described in Table 6.1. An air filtering system operated in ward E only. All other wards were ventilated naturally. Both HCWs and patients were approached by a research nurse, asked to participate in the study and provided with the relevant information leaflet (Appendix 2). Those who provided informed consent to participate in the study filled-in and signed the relevant consent form (Appendix 3). Healthcare workers were defined as staff members with any form of direct patient contact, including housekeeping (i.e. catering and cleaning) and clerical staff. Allied healthcare professionals were defined as dieticians, occupational therapists, physiotherapists and speech therapists. Power calculations were performed to determine that the recruitment of 127 patients and 146 HCWs was required in order to determine the prevalence of *S. aureus* in each group with a margin of error of ±7.5%. Volunteers were requested to complete a detailed questionnaire, as shown in Figure 6.1.

6.2.3 Participant, environmental and air sampling

The research nurse sampled the anterior nares of each participant using a sterile transport swab (Sarstedt, Wexford, Ireland). Oral rinses were also collected by providing each participant with 25 ml of sterile phosphate-buffered saline (PBS) in a 100 ml disposable cup (Sarstedt) and instructing them to rinse the PBS in their mouth for 30 s before returning it to the cup and screwing the cap closed. Near-patient environmental sampling was performed by rotating a sterile transport swab (Sarstedt), in three different directions, across a 10 cm^2 area of each patient's bed frame, bedside locker and mattress. An Oxoid EM0100A air sampler (Fisher Scientific, Dublin, Ireland) was used to sample 1000 L of air in 6-21 different locations per ward, depending on the size of the ward and access

Table 6.1 Details of the nine hospital wards in which healthcare workers and patients were recruited over a six-month period in 2017

Ward	Description	No. of beds				Floor No.
		Open-ward[a]	Two-bed room	Single room	Total	
A	General, vascular and upper gastroenterology surgery	28	2	5	35	4
B	Orthopaedic surgery	28	2	5	35	4
C	Geriatric medicine	28	2	5	35	3
D	Infectious diseases and general medicine	28	2	5	35	3
E	Transplant and nephrology surgery	9	0	12	21	4
F	Gastroenterology medicine	28	2	5	35	2
G	Urology surgery	12	2	2	16	1
H	Respiratory medicine	28	2	5	35	2
I	Renal medicine	26	2	5	33	4
Total	NA	215	16	49	280	NA

[a]Open-ward beds were defined as those in bays of four or more beds.

Abbreviation: NA, not applicable.

A.

HEALTHCARE WORKER QUESTIONNAIRE

An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

TIME **DATE** **WARD**

PLEASE ANSWER EACH OF THE FOLLOWING QUESTIONS

1. Age range (years):

18-24 □ 25-34 □ 35-44 □ 45-54 □ 55-64 □ 65 or older □

2. Gender: Male □ Female □

3. Country of birth: _____

4. If born abroad, how long have you been living in Ireland:

<1 year □ 1-5 years □ 6-10 years □ >10 years □

5. Role in hospital: _____

6. Number of hours of patient contact had per day:

0-30 mins □ 30 mins-1 hour □

1-2 hours □ 2-4 hours □ 4-8 hours □ 8-12 hours □ >12 hours □

7. Ward type: _____

8. Length of employment in Beaumont Hospital:

<1 year □ 1-5 years □ 6-10 years □ >10 years □

9. Previous hospital employment:

Yes □ No □

10. If YES, was that in: Another Irish hospital. □ A hospital abroad □

If abroad, where? _____

11. Have you been hospitalised overnight for any reason in the last year?

Yes □ No □

12. If yes, were you hospitalized in:

This hospital □ Another Irish hospital □ Nursing Home □

Residential Unit □ Rehabilitation Unit □ A hospital abroad □

If abroad, where: _____ or Not Applicable □

13. Have you taken steroids during the past year? Yes □ No □

14. Have you taken antibiotics during the past year? Yes □ No □

15. Have you travelled abroad during the past year? Yes □ No □

16. If YES, where was that travel to?

Within Europe □ Middle East □ Far East □ Australia and New Zealand □
Asia □ South America □ Central America □ USA □
Other_____

17. Have you had contact with farm animals in the last year? Yes □ No □

18. Do you have a history of the following? (Please answer 'yes', 'no', 'unknown'.)

a) Abscesses _____
b) Boils
_____
c) Bone infections _____
d) Septic arthritis _____
e) Necrotizing pneumonia _____
f) Cellulitis _____
g) Other skin conditions _____

B.

PATIENT QUESTIONNAIRE

An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

TIME **DATE** **WARD**

PLEASE ANSWER EACH OF THE FOLLOWING QUESTIONS

1. Age range (Years):

   18-24 □ 25-34 □ 35-44 □
   45-54 □ 55-64 □ 65 or older □

2. Gender: Male □ Female □

3. Type of hospital room: Single side room □ Double side room □ Open ward bay □

4. Ward Type: _____

5. Country of Birth: _____

6. If born abroad, how long have you been living in Ireland:

   <1 year □ 1-5 years □ 6-10 years □ >10 years □

7. Length of current hospitalisation: 0-24 hours □ 24-48 hours □ 2-7 days □ 1-2 weeks □ >2 weeks □
8. Previous hospitalisation for more than 24 hours in the past year? Yes □ No □

9. If YES was that hospitalisation in:
   This hospital □ Another Irish hospital □ Nursing Home □
   Residential Unit □ Rehabilitation Unit □ A hospital abroad □
   If abroad, where: _____, or Not Applicable □

10. Have you taken antibiotics during the last year? Yes □ No □

11. Have you taken steroids during the last year? Yes □ No □

12. Have you travelled abroad during the last year? Yes □ No □

    If YES, where was that travel to? Within Europe □ Middle East □ Far East □ Australia and New Zealand □ Asia □ South America □ Central America □ USA □ Other_____

13. Have you had contact with farm animals in the last year? Yes □ No □

14. Do you have a history of the following? (Please answer ‘yes’, ‘no’, ‘unknown’.)

a) Abscesses _____
b) Boils _____
c) Bone infections _____
d) Septic arthritis _____
e) Necrotizing pneumonia _____
f) Cellulitis _____
g) Other skin conditions _____

Figure 6.1 The questionnaires with which (A) healthcare worker and (B) patient participants were provided during the present study.

constraints. Separate Sa*Select* and MRSA*Select* chromogenic agar plates (Bio-Rad) were each used to sample 500 L of air at each location. All samples were stored at 4ºC and transported to the Dublin Dental University Hospital (DDUH) within 24 h.

6.2.4 Sample processing

Upon arrival at DDUH, the air sampling plates were incubated for 48 h at 37ºC. The remaining samples were processed within 48 h of arrival. All swabs were processed as described in Chapter 2, section 2.3.1. In brief, each swab was used to lawn both an Sa*Select* and an MRSA*Select* chromogenic agar plate, prior to incubation for 48 h at 37ºC. To ensure maximum bacterial recovery, plates were lawned in three different directions, while rotating the swab. Oral rinses were vortexed in their collection cups for 30 s at maximum speed, using a Heidolph Reax vortex (Heidolph Instruments GmbH & Co., Schwabach, Germany). Immediately following vortexing, 1 ml volumes of each oral rinse was transferred into a sterile 1.5 ml Safelock microfuge tube (Eppendorf). The 1.5 ml tubes were then centrifuged in an Eppendorf model 5417C bench top centrifuge at 20,000 x g for 1 min. The supernatant from each sample was discarded and the pellet was resuspended in 300 µl of PBS. Separate 100 µl volumes of each suspension were plated onto Sa*Select* and MRSA*Select* chromogenic agar plates. These plates were incubated for 48 h at 37ºC.

Isolates were confirmed as *S. aureus* using the tube coagulase test, as described in Chapter 2, section 2.3.2. Isolates were confirmed as MRSA or MSSA using 30-µg cefoxitin disks, as described in Chapter 2, section 2.3.3. For the HCW, patient and environmental samples, if *S. aureus* was identified on both the Sa*Select* and the MRSA*Select* chromogenic agar plate, growth from the MRSA*Select* plate only, was processed further. Two isolates (one oral and one nasal) were stored for participants who yielded both oral and nasal *S. aureus*. One isolate was stored for participants who yielded either nasal or oral *S. aureus*. One isolate was stored for each environmental site (i.e. bed frame, bedside locker and mattress) that yielded *S. aureus*. A maximum of four isolates from each agar plate were stored per air sampling site. Isolates were stored as described in Chapter 2, section 2.2.1.

6.2.5 Clinical isolate collection

All available SSTI *S. aureus* isolates recovered from patients on the study wards during the six-month study period were collected from the Beaumont Hospital Clinical Microbiology Laboratory. All BSI *S. aureus* isolates recovered on the study wards in 2017 were also included in this study. For consistency, these isolates were processed as described in

section 6.2.4, i.e. isolates were subcultured onto both Sa*Select* and MRSA*Select* chromogenic agar, confirmed as *S. aureus* using the tube coagulase test and confirmed as MSSA or MRSA using 30-µg cefoxitin disks.

6.2.6 Antimicrobial susceptibility testing

All isolates underwent antimicrobial susceptibility testing against a panel of 20 antimicrobial agents, as described in Chapter 2, section 2.3.4. The prevalence of each resistance phenotype was assessed separately among the MRSA and MSSA isolates and represented visually using a clustered bar chart constructed in Microsoft Excel (version 16.16.3). If a single air sample site yielded two or more isolates exhibiting identical antimicrobial susceptibility profiles, only one such isolate was retained for further analysis.

6.2.7 WGS

All patient, HCW, environmental and clinical isolates, and all air isolates selected for further analysis, underwent Illumina-MiSeq WGS as described in Chapter 2, section 2.5 (Table 6.2).

6.2.8 *de novo* assembly

Sequence read sets were assembled into contigs using SPAdes (Bankevich et al., 2012), as described in Chapter 2, section 2.6.2.

6.2.9 wgMLST

Each isolate genome was analysed using the wgMLST scheme available in BioNumerics v7.6 (Applied Maths), as described in Chapter 2, section 2.6.8. The data generated using this algorithm were used during population structure analysis (section 6.2.10) and cluster identification (section 6.2.12).

6.2.10 Analysis of the population structure of *S. aureus* on the study wards

In order to facilitate inter-study comparisons, conventional MLST was used to assess the population structure of *S. aureus* on the study wards. The contig sets of all isolates underwent MLST using SeqSphere+ version 4.1 (Ridom GmbH), as described in Chapter 2, section 2.6.3. While assessing the prevalence of each ST among the study participants, one isolate was included per participant unless an individual yielded isolates with two different STs. Using these selected participant isolates, BioNumerics v7.6 (Applied Maths) was used to construct a MST based on the seven traditional MLST loci only, as described

Table 6.2 Whole-genome sequencing quality assurance data

Quality parameter	Average result
Trimmed read quality	37.1
N50[a]	384002.7
No. of contigs per isolate	32.8
Assembly coverage	128.4
% core-genome loci present[b]	98.9

[a]The minimum contig length required to cover half the genome.
[b]According to the previously defined core-genome multilocus sequence typing scheme (Leopold et al., 2014).

in Chapter 2, section 2.6.10. Isolate contig sets also underwent *spa* typing, as described in Chapter 2, section 2.6.4. For 18 study isolates for which WGS-based *spa* typing failed, conventionally *spa* typing was performed as described in Chapter 2, section 2.4.3.

6.2.11 Isolate genotyping

All isolates underwent *in silico* genotyping using SeqSphere+ version 4.1 (Ridom GmbH) to (i) identify the resistance and virulence-associated genes present in each isolate and (ii) determine the SCC*mec* type of each MRSA isolate. In SeqSphere+, the complete coding sequences of the target genes were searched using the BLAST algorithim (Altschul et al., 1990). Targets were regarded as present if they exhibited ≥ 95% sequence identity and ≥ 99% query cover to the reference sequences stored in SeqSphere+. If multiple matches were identified for a single target, the best match was selected. Targets including internal stop codons, frame shifts, or nucleotide ambiguities were included. Partial targets were not included. The prevalence of each resistance and virulence-associated gene was assessed per ST and represented visually using a stacked bar chart constructed in Microsoft Office Excel.

6.2.12 Identification of intra-hospital transmission events

A transmission event was defined as the transfer of *S. aureus* between two individuals, or between an individual and the air or environment. A wgMLST distance matrix was generated in BioNumerics v7.6 (Applied Maths) and exported into Excel for analysis. This distance matrix included all isolates that underwent WGS. In order to assess isolate relatedness and thus, identify recent transmission events, data interpretation guidelines (see results, section 6.3.7.1) were developed based, in part, on three different aspects of these wgMLST data. Firstly, the wgMLST data set was assessed as a unit. Secondly, in order to gauge the impact of *in vivo* strain variation on the data, the variation between isolates of the same ST recovered from single participants, was investigated. Finally, in order to estimate the extent to which isolates recently transmitted from a common source may vary, all instances were examined in which two or more isolates of the same ST were recovered in the same patient area. Previous studies were also considered during development of data interpretation guidelines. Specifically, it was noted that a relatedness threshold of ≤ 24 wgMLST allelic differences was tentatively proposed for *S. aureus*, recently (Schürch et al., 2018). The wgMLST data were visually represented using MSTs constructed in BioNumerics v7.6 (Applied Maths), as described in Chapter, section 2.6.2.10.

6.2.13 Data management and statistical analysis

All participant data were pseudonymised and descriptive coding systems were developed for all samples collected. All epidemiological, prevalence, phenotypic and genotypic data were amalgamated into a single Excel file. A data dictionary was created in Microsoft Word (v16.16.3; Microsoft Office) and a number-coded version of the amalgamated data set was generated. This number-coded data set was imported into the SPSS Statistics (v25.0.0.1; International Business Machines Corporation, New York, U.S.A) software package for statistical analysis. The Chi-Square Test was used to compare categorical variables. The Mann-Whitney U test was used to compare independent groups, when the dependent variable was continuous or ordinal. The Spearman's Rho test was used to examine the correlation between continuous variables. Differences between groups were considered statistically significant if $p < 0.05$. Correlation between groups was considered very weak if $r_s = 0.00$ to ± 0.19, weak if $r_s = \pm 0.20$ to ± 0.39, moderate if $r_s = \pm 0.40$ to ± 0.59, strong if $r_s = \pm 0.60$ to ± 0.79 and very strong if $r_s = \pm 0.80$ to ± 1.00.

6.3 Results

6.3.1 Description of sample group

A total of 281 volunteers took part in the present study. This included 149 HCWs and 132 hospital inpatients.

Over half (58.4%; 87/149) of the HCW participants were nurses, 15.4% (23/149) were healthcare assistants, 12.8% (19/149) were medical personnel, 8.7% (13/149) were allied healthcare professionals, 2.7% (4/149) were clerical workers and 2.0% (3/149) were housekeepers. The majority (83.9%; 125/149) of HCW participants were female. The 25-34 y age-bracket included the highest proportion (44.3%; 66/149) of HCW participants, followed in order by 35-44 y (22.2%; 34/149), 18-24 y (22.1%; 33/149), 45-54 y (7.4%; 11/149) and 55-64 y (3.6%; 5/149). On average, both healthcare assistants and nurses reported the highest number of hours per working day of direct patient contact (5.3 h), followed in order by allied healthcare professionals (4.1 h), medical personnel (3.8 h), housekeepers (2.3 h) and clerical workers (1.3 h). HCW participants had been employed at the hospital an average of 2.2 (min = 1; max = 4) years at the time of sampling and 57.7% (86/149) had been previously employed in a different hospital/HCF. Within the 12 months prior to sampling, the majority (79.2%; 118/149) of HCW participants had travelled abroad, 26.2% (39/149) had had contact with farm animals and 12.1% (18/149) had taken antibiotics. A small proportion (6.0%; 9/149) of HCWs had a history of SSTIs. Approximately one third (34.2%; 51/149) of HCW participants were of non-Irish ethnic origin. India was by far the most common (49.0%; 25/51) alternative country of ethnic origin, followed in order by the Philippines (15.7%; 8/51) and the UK (7.8%; 4/51).

The 65+ years age-bracket included the highest proportion (38.6%; 51/132) of patient participants, followed in order by 55-64 y (26.5%; 35/132), 45-54 y (17.4%; 23/132), 35-44 y (7.6%; 10/132), 25-34 y (5.3%; 7/132) and 18-24 y (4.5%; 6/132). The majority (78.0%; 103/132) of patient participants were in open-ward beds, while 7.6% (10/132) were in two-bed rooms, 12.9% (17/132) were in single rooms and 1.5% (2/132) were in extra-capacity beds in ward corridors. A majority (56.1%; 74/132) of patient participants were male. At the time of sampling, the patient participants had been in hospital for an average of nine (min = 1; max = 73) days. Within the 12 months prior to sampling, and excluding their current hospital stay, 56.8% (75/132) patient participants had been admitted into either the study hospital or a different hospital/HCF. Furthermore, during the 12 months prior to sampling, 40.2% (53/132) of patient participants had travelled abroad,

19.7% (26/132) had contact with farm animals and 73.5% (97/132) had taken antibiotics. A large proportion (59.1%; 78/132) of patients had a history of SSTIs, while 12.9% (17/132) had a history of invasive infections. The vast majority (90.2%; 119/132) of patient participants were of Irish ethnic origin.

6.3.2 Description of isolates investigated

A total of 195 *S. aureus* isolates were investigated, including 170 MSSA and 25 MRSA (Table 6.3). In detail, 64 HCW isolates were investigated from 53 different HCWs. This included one isolate from each of 42 HCWs who yielded either a nasal or oral isolate, and two isolates from each of 11 HCWs who yielded both nasal and oral isolates. Forty patient isolates were investigated from 30 different patients. This included one isolate from each of 20 patients who yielded either a nasal or oral isolate, and two isolates from each of 10 patients who yielded both nasal and oral isolates. Thirty environmental isolates were investigated from 20 different patient areas. Twelve, six and two of these patient areas yielded one, two and three isolates, respectively. Thirty-five air isolates were investigated from 28 different air sampling sites. This included one isolate from each of 22 sites, two isolates from each of five sites and three isolates from one site. Finally, 26 clinical isolates were investigated, 10 of which were recovered from SSTIs and 16 of which were recovered from BSIs.

6.3.3 *Staphylococcus aureus* prevalence

The prevalence of *S. aureus* per ward and sample group (i.e. HCW, patient, environment and air) is detailed in Table 6.4.

Staphylococcus aureus was recovered from 29.5% (83/281) of all study participants. Nasal isolates only were recovered from 51% (43/83) of *S. aureus*-positive participants, while oral isolates only were recovered from 23% (19/83) of *S. aureus*-positive participants. Both oral and nasal isolates were recovered from the remaining 25% (21/83) of *S. aureus*-positive participants. Significantly more HCWs (35.6%; 53/149) harboured *S. aureus* compared to patients (22.7%; 30/132; $p = 0.019$). Carriage was not associated with gender ($p = 0.573$), age ($p = 0.303$), recent travel ($p = 0.505$) or recent contact with farm animals ($p = 0.951$). However, *S. aureus* prevalence was significantly lower among those who had recently taken antibiotics ($p = 0.034$). MRSA was recovered from 2.5% (7/281) of all study participants and 8.4% (7/83) of *S. aureus*-positive participants. MRSA carriage was higher among HCWs (3.4%; 5/149) compared to patients (1.5%; 2/132), however,

Table 6.3 The epidemiological and conventional molecular typing data associated with the 195 HCW, patient, environmental, air and clinical *S. aureus* isolates recovered on nine wards of a large Dublin hospital in 2017

Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]	Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]
HN0020	HCW	Nasal	B	MSSA	25	t081	HN0200	HCW	Nasal	C	MSSA	5	t088
HN0024	HCW	Nasal	G	MSSA	398	t1451	HN0202	HCW	Nasal	C	MSSA	320	t084
HN0034	HCW	Nasal	B	MSSA	30	t012	HN0206	HCW	Nasal	C	MSSA	5033	t346
HN0036	HCW	Nasal	B	MRSA	22	t032	HN0214	HCW	Nasal	H	MRSA	22	t032
HN0044	HCW	Nasal	B	MSSA	6	t304	HN0216	HCW	Nasal	H	MSSA	8	t2842
HN0072	HCW	Nasal	E	MSSA	34	t3728	HN0218	HCW	Nasal	H	MSSA	45	t026
HN0076	HCW	Nasal	E	MSSA	30	t021	HN0220	HCW	Nasal	F	MSSA	45	t230
HN0094	HCW	Nasal	I	MSSA	508	t4001	HN0224	HCW	Nasal	H	MSSA	30	t012
HN0104	HCW	Nasal	I	MSSA	5042	t334	HN0232	HCW	Nasal	F	MSSA	45	t102
HN0118	HCW	Nasal	C	MSSA	30	t14734	HN0264	HCW	Nasal	D	MSSA	45	t015
HN0128	HCW	Nasal	C	MSSA	34	t166	HN0278	HCW	Nasal	H	MSSA	22	t4640
HN0132	HCW	Nasal	A	MSSA	2233	t18376	HN0280	HCW	Nasal	H	MSSA	45	t015
HN0138	HCW	Nasal	C	MSSA	582	t084	HN0282	HCW	Nasal	H	MSSA	30	t342
HN0140	HCW	Nasal	A	MSSA	96	t359	HN0284	HCW	Nasal	H	MSSA	30	t019
HN0142	HCW	Nasal	A	MSSA	30	t021	HO0016	HCW	Oral	B	MSSA	972	t230
HN0144	HCW	Nasal	C	MSSA	22	t005	HO0024	HCW	Oral	G	MSSA	5	t548
HN0164	HCW	Nasal	C	MSSA	672	t3841	HO0030	HCW	Oral	E	MSSA	582	t084
HN0166	HCW	Nasal	C	MSSA	5	t2065	HO0034	HCW	Oral	B	MSSA	8	t2293
HN0172	HCW	Nasal	F	MSSA	789	t1243	HO0036	HCW	Oral	B	MSSA	22	t032
HN0184	HCW	Nasal	F	MSSA	6	t304	HO0072	HCW	Oral	E	MSSA	34	t3728
HN0186	HCW	Nasal	F	MSSA	508	t827	HO0084	HCW	Oral	I	MSSA	30	t012
HN0188	HCW	Nasal	F	MSSA	398	t571	HO0094	HCW	Oral	I	MSSA	508	t4001
HN0194	HCW	Nasal	F	MRSA	5	t311	HO0102	HCW	Oral	A	MRSA	5	t311
HN0198	HCW	Nasal	F	MSSA	45	t620	HO0130	HCW	Oral	A	MRSA	6	t11288

Table 6.2 continued overleaf

Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]	Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]
HO0150	HCW	Oral	A	MSSA	3634	t026	PN0079	Patient	Nasal	E	MSSA	8	t18374
HO0168	HCW	Oral	F	MSSA	30	t012	PN0083	Patient	Nasal	I	MSSA	5	t002
HO0194	HCW	Oral	F	MRSA	5	t311	PN0085	Patient	Nasal	I	MSSA	45	t550
HO0214	HCW	Oral	H	MSSA	22	t032	PN0089	Patient	Nasal	I	MSSA	45	t230
HO0220	HCW	Oral	F	MSSA	45	t230	PN0101	Patient	Nasal	A	MSSA	5043	t012
HO0234	HCW	Oral	F	MSSA	45	t004	PN0119	Patient	Nasal	A	MSSA	20	t164
HO0236	HCW	Oral	F	MSSA	5	t9663	PN0127	Patient	Nasal	A	MSSA	5	t002
HO0244	HCW	Oral	H	MSSA	15	t228	PN0129	Patient	Nasal	A	MSSA	30	t018
HO0248	HCW	Oral	H	MSSA	45	t004	PN0131	Patient	Nasal	A	MSSA	34	t089
HO0252	HCW	Oral	D	MSSA	1160	t6140	PN0143	Patient	Nasal	A	MSSA	5	t548
HO0256	HCW	Oral	D	MSSA	1637	t002	PN0173	Patient	Nasal	F	MSSA	54	t157
HO0258	HCW	Oral	H	MSSA	5031	t230	PN0205	Patient	Nasal	F	MSSA	1	t127
HO0278	HCW	Oral	H	MSSA	5036	t026	PN0217	Patient	Nasal	F	MRSA	5	t311
HO0280	HCW	Oral	H	MSSA	45	t015	PN0223	Patient	Nasal	D	MSSA	30	t18404
HO0284	HCW	Oral	H	MSSA	30	t021	PN0227	Patient	Nasal	D	MSSA	5031	t230
HO0286	HCW	Oral	H	MSSA	45	t230	PN0245	Patient	Nasal	D	MSSA	45	t026
PN0001	Patient	Nasal	G	MSSA	398	t1451	PO0001	Patient	Oral	G	MSSA	398	t1451
PN0013	Patient	Nasal	G	MSSA	15	t18403	PO0023	Patient	Oral	B	MSSA	34	t136
PN0023	Patient	Nasal	B	MSSA	34	t136	PO0033	Patient	Oral	B	MSSA	1	t127
PN0033	Patient	Nasal	B	MSSA	22	t2251	PO0041	Patient	Oral	B	MSSA	5041	t084
PN0035	Patient	Nasal	B	MSSA	30	t138	PO0043	Patient	Oral	B	MSSA	8	t008
PN0041	Patient	Nasal	B	MSSA	5041	t084	PO0063	Patient	Oral	I	MSSA	15	t18373
PN0043	Patient	Nasal	B	MSSA	8	t008	PO0065	Patient	Oral	I	MSSA	45	t362
PN0057	Patient	Nasal	E	MSSA	30	t7561	PO0083	Patient	Oral	I	MSSA	5	t002
PN0063	Patient	Nasal	I	MSSA	15	t18373	PO0107	Patient	Oral	I	MSSA	5034	t18375
PN0065	Patient	Nasal	I	MSSA	45	t362	PO0157	Patient	Oral	A	MSSA	22	t15630

Table 6.2 continued overleaf

Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]	Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]
PO0161	Patient	Oral	A	MSSA	45	t015	EL0161	Environ	Locker	A	MSSA	5	t548
PO0185	Patient	Oral	F	MRSA	22	t032	EL0167	Environ	Locker	A	MRSA	5	t548
PO0227	Patient	Oral	D	MSSA	5031	t230	EL0169	Environ	Locker	A	MSSA	5	t548
PO0245	Patient	Oral	D	MSSA	45	t026	EL0205	Environ	Locker	F	MSSA	1	t127
EB0033	Environ	Bed frame	B	MSSA	1	t127	EM0145	Environ	Mattress	A	MSSA	5	t548
EB0083	Environ	Bed frame	I	MSSA	5	t002	EM0161	Environ	Mattress	A	MSSA	5	t548
EB0101	Environ	Bed frame	A	MSSA	5043	t012	AAB0036B	Air	NA	A	MSSA	5	t548
EB0113	Environ	Bed frame	A	MSSA	5	t548	AAB0042A	Air	NA	A	MRSA	22	t032
EB0117	Environ	Bed frame	A	MSSA	188	t5872	AAB0042B	Air	NA	A	MSSA	5	t548
EB0131	Environ	Bed frame	A	MSSA	5	t548	AAB0043	Air	NA	A	MRSA	5	t548
EB0143	Environ	Bed frame	A	MSSA	5	t548	AAB0045	Air	NA	A	MSSA	5	t548
EB0151	Environ	Bed frame	A	MSSA	5	t548	AAB0047A	Air	NA	A	MSSA	5	t548
EB0155	Environ	Bed frame	A	MSSA	5	t548	AAB0049	Air	NA	A	MSSA	30	t338
EB0161	Environ	Bed frame	A	MSSA	5	t548	ABA0013A	Air	NA	B	MSSA	34	t136
EB0169	Environ	Bed frame	A	MSSA	5	t548	ABA0016A	Air	NA	B	MRSA	5	t311
EB0205	Environ	Bed frame	F	MSSA	1	t127	ABA0016B	Air	NA	B	MSSA	45	t445
EB0227	Environ	Bed frame	D	MSSA	5031	t230	ADA0022	Air	NA	E	MRSA	22	t1612
EL0015	Environ	Locker	G	MSSA	398	t1451	AHA0053A	Air	NA	C	MSSA	5033	t346
EL0027	Environ	Locker	B	MSSA	398	t1255	AHA0054	Air	NA	C	MSSA	5033	t346
EL0031	Environ	Locker	G	MSSA	398	t1451	AHA0065	Air	NA	C	MSSA	5033	t346
EL0033	Environ	Locker	B	MSSA	1	t127	AJE0094	Air	NA	D	MSSA	45	t065
EL0059	Environ	Locker	I	MSSA	20	t1544	AJE0102	Air	NA	D	MSSA	5	t688
EL0063	Environ	Locker	I	MSSA	5029	t9228	AJE0103	Air	NA	D	MSSA	1027	t647
EL0113	Environ	Locker	A	MSSA	5	t548	AJE0105A	Air	NA	D	MSSA	22	t223
EL0145	Environ	Locker	A	MSSA	5	t548	AJE0105B	Air	NA	D	MSSA	398	t571
EL0151	Environ	Locker	A	MSSA	5	t548	AJE0106A	Air	NA	D	MSSA	398	t571

Table 6.2 continued overleaf

Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]	Isolate no.[a]	Isolate source	Site sampled	Ward	MSSA/ MRSA	Sequence type[b]	*spa* type[c]
AJE0108	Air	NA	D	MSSA	34	t16237	17M0092946	Clinical	BSI	A	MSSA	45	t015
AJE0109	Air	NA	D	MSSA	1	t693	17M0099331	Clinical	Wound	C	MRSA	22	t1612
AJE0111	Air	NA	D	MRSA	22	t032	17M0100638	Clinical	BSI	F	MRSA	22	t020
AJE0113A	Air	NA	D	MSSA	398	t571	17M0101093	Clinical	BSI	I	MSSA	59	Unknown[d]
ALA0084	Air	NA	F	MSSA	2383	t2279	17M0104066	Clinical	Wound	A	MRSA	22	t1612
ALA0094	Air	NA	F	MSSA	5039	t002	17M0104123	Clinical	Wound	B	MRSA	22	t032
AMA0002A	Air	NA	G	MSSA	5	t088	17M0104218	Clinical	Wound	I	MSSA	30	t3732
AMA0002B	Air	NA	G	MRSA	22	t020	17M0106663	Clinical	Wound	G	MSSA	5	t442
AMA0002C	Air	NA	G	MSSA	398	t1451	17M0111576	Clinical	Wound	A	MRSA	1027	t647
AMA0004A	Air	NA	G	MSSA	398	t1451	17M0112296	Clinical	BSI	G	MSSA	1027	t647
AMA0005A	Air	NA	G	MSSA	5030	t160	17M0114756	Clinical	Wound	I	MSSA	22	t1214
AMA0005B	Air	NA	G	MSSA	22	t1802	17M0117117	Clinical	Wound	F	MSSA	5	t088
AMA0006A	Air	NA	G	MRSA	398	t1451	17M0119224	Clinical	Wound	C	MSSA	5	t548
AMA0006B	Air	NA	G	MSSA	22	t020	17M0123011	Clinical	Wound	D	MSSA	15	t084
APA0096	Air	NA	H	MRSA	22	t032	17M0124932	Clinical	BSI	A	MRSA	5040	t008
17M0000079	Clinical	BSI	F	MSSA	5	t002	17M0160277	Clinical	BSI	A	MSSA	109	t209
17M0013278	Clinical	BSI	D	MSSA	30	t1515	17M0165383	Clinical	BSI	I	MRSA	22	t032
17M0032837	Clinical	BSI	B	MSSA	582	t094	17M0166108	Clinical	BSI	F	MSSA	109	t209
17M0037724	Clinical	BSI	H	MSSA	5039	t548	17M0171546	Clinical	BSI	I	MSSA	2889	t4328
17M0072785	Clinical	BSI	B	MSSA	7	t091	17M0171679	Clinical	BSI	D	MSSA	398	t18379
17M0075806	Clinical	BSI	A	MSSA	45	t16965	17M0092946	Clinical	BSI	A	MSSA	45	t015

Footnotes overleaf

[a]HCW and patient isolates with the same last four digits were recovered from the same individual. The last four digits of an environmental isolate indicates the patient with which it is associated. Air isolates with the same last four digits (excluding the letter at the end of some air isolates codes) were recovered from the same air site.

[b]Traditional MLST sequence types were assigned by analysing WGS contig sets using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany).

[c]*spa* typing was performed either conventionally, as described in Chapter 2, section 2.4.3, or by analysing WGS contig sets using Ridom SeqSphere+ version 4.1 (Ridom GmbH).

[d]*spa* typing repeatedly failed for this isolate, using both of the methods described above.

Abbreviations: BSI, bloodstream infection; environ, environmental; HCW, healthcare worker; NA, not applicable; WGS, whole-genome sequencing.

Table 6.4 MSSA and MRSA prevalence per ward and sample group

Ward	Healthcare workers[a]			Patients[a]			Environmental areas[b]			Air sites[c]			
	No. of MSSA+ (%)	No. of MRSA+ (%)	Total (%)	No. of MSSA+ (%)	No. of MRSA+ (%)	Total (%)	No. of MSSA+ (%)	No. of MRSA+ (%)	Total (%)	No. of MSSA+ only (%)	No. of MRSA+ only (%)	No. of MRSA and MSSA+ (%)	Total (%)
A	4/12 (33.3)	2/12 (16.6)	6/12 (50.0)	8/29 (27.6)	0/29 (0.0)	8/29 (27.6)	11/29 (38.0)	0/29 (0.0)	11/29 (38.0)	5/20 (25.0)	0/20 (0.0)	1/20 (5.0)	6/20 (30.0)
B	4/13 (30.8)	1/13 (7.7)	5/13 (38.5)	5/13 (38.5)	0/13 (0.0)	5/13 (38.5)	2/13 (15.4)	0/13 (0.0)	2/13 (15.4)	1/10 (10.0)	0/10 (0.0)	1/10 (10.0)	2/10 (20.0)
C	9/23 (39.1)	0/23 (0.0)	9/23 (39.1)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	3/20 (15.0)	0/20 (0.0)	0/20 (0.0)	3/20 (15.0)
D	4/14 (28.6)	0/14 (0.0)	4/14 (28.6)	3/12 (25.0)	0/12 (0.0)	3/12 (25.0)	1/12 (8.3)	0/12 (0.0)	1/12 (8.3)	8/20 (40.0)	1/20 (5.0)	0/20 (0.0)	9/20 (45.0)
E	3/8 (37.5)	0/8 (0.0)	3/8 (37.5)	2/11 (18.2)	0/11 (0.0)	2/11 (18.2)	0/11 (0.0)	0/11 (0.0)	0/11 (0.0)	0/8 (0.0)	1/8 (12.5)	0/8 (0.0)	1/8 (12.5)
F	10/25 (40.0)	1/25 (4.0)	11/25 (44.0)	2/20 (10.0)	2/20 (10.0)	4/20 (20.0)	1/20 (5.0)	0/20 (0.0)	1/20 (5.0)	2/21 (9.5)	0/21 (0.0)	0/21 (0.0)	2/21 (9.5)
G	1/10 (10.0)	0/10 (0.0)	1/10 (10.0)	2/10 (20.0)	0/10 (0.0)	2/10 (20.0)	2/10 (20.0)	0/10 (0.0)	2/10 (20.0)	1/6 (16.6)	1/6 (16.6)	2/6 (33.3)	4/6 (66.7)
H	10/21 (47.6)	1/21 (4.8)	11/21 (52.4)	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)	0/13 (0.0)	0/20 (0.0)	1/20 (5.0)	0/20 (0.0)	1/20 (5.0)
I	3/23 (13.0)	0/23 (0.0)	3/23 (13.0)	6/19 (31.6)	0/19 (0.0)	6/19 (31.6)	3/19 (15.8)	0/19 (0.0)	3/19 (15.8)	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)
Total	48/149 (32.2)	5/149 (3.4)	53/149 (35.6)	28/132 (21.2)	2/132 (1.5)	30/132 (22.7)	20/132 (15.2)	0/132 (0.0)	20/132 (15.2)	20/135 (14.8)	4/135 (3.0)	4/135 (3.0)	28/135 (20.7)

Footnotes overleaf

[a]Both nasal and oral samples were obtained for each participant. Prevalence rates per recovery site are detailed in section 6.3.3. Each sample was processed using both Sa*Select* and MRSA*Select* chromogenic agar (Bio-Rad). If *S. aureus* was identified on both types of agar, growth from the MRSA*Select* plate only, was considered.

[b]A single patient area included the patient's bedframe, bedside locker and mattress. Prevalence rates per recovery site are detailed in section 6.3.3. Each sample was processed using both Sa*Select* and MRSA*Select* chromogenic agar (Bio-Rad). If *S. aureus* was identified on both types of agar, growth from the MRSA*Select* plate only, was considered.

[c]Air sampling was performed using an Oxoid EM0100A air sampler. A total of 1000 L was sampled per site, using Sa*Select* (500 L) and MRSA*Select* (500 L) chromogenic agar plates (Bio-Rad).

this difference was not significant ($p = 0.323$).

Staphylococcus aureus was detected among all five HCW roles. Specifically, 30% (26/87) of nurses, 48% (11/23) of healthcare assistants, 37% (7/19) of medical personnel, 46% (6/13) of allied healthcare professionals, 50% (2/4) of clerical workers and 67% (2/3) of housekeepers harboured *S. aureus*. Carriage rates were not significantly higher in any particular employment category compared to the remaining categories. The five HCW MRSA isolates were recovered from three nurses, one healthcare assistant and one housekeeper. Healthcare workers from all nine wards yielded *S. aureus*, as detailed in Table 6.4. Carriage of *S. aureus* was significantly lower among HCWs in ward I compared to the remaining wards ($p = 0.014$). There were no other significant differences between wards in relation to *S. aureus* carriage among HCWs.

Staphylococcus aureus was recovered from 15.2% (20/132) of all patient environmental areas tested. Specifically, 9.8% (13/132) of bed frames and of bedside lockers, and 3.0% (4/132) of mattresses, yielded *S. aureus*. None of these sites yielded MRSA. The rate of near-patient environmental contamination per ward was positively associated with *S. aureus* carriage among patients, however, this association was not quite significant ($p = 0.058$). In contrast, the rate of near-patient environmental contamination per ward was negatively associated with *S. aureus* carriage among HCWs ($p = 0.050$). Aerial contamination was detected in 20.7% (28/135) of sites tested. MSSA only and MRSA only were detected at 14.8% (20/135) and 3.0% (4/135) of air sampling sites, respectively. Both MSSA and MRSA were detected at 3.0% (4/135) of air sampling sites. The rate of aerial contamination per ward was not significantly associated with *S. aureus* prevalence among patients ($p = 0.448$) or HCWs ($p = 0.731$). A moderate positive correlation ($r_s = 0.429$) was observed between aerial and near-patient environmental contamination per ward and this correlation was significant ($p < 0.001$).

6.3.4 Population structure of *S. aureus* on the study wards

A total of 42 different STs and 85 different *spa* types were identified among the 195 isolates investigated (Tables 6.2 and 6.4). The distribution of these STs among the HCWs, patients, patient areas, air sampling sites and clinical isolates is shown in Table 6.5. Isolate *spa* types are shown in Table 6.3 only. Overall, ST5 (20.5%; 40/195) was the most

Table 6.5 The traditional MLST sequence types identified among 195 HCW, patient, environmental, air and clinical *S. aureus* isolates recovered on nine wards of a large Dublin hospital in 2017

Sequence type[a]	No. of isolates (%)	HCW		Patient		Environmental		Air		Clinical
		No. of HCWs (%)	No. of isolates (%)	No. of patients (%)	No. of isolates (%)	No. of areas (%)	No. of isolates (%)	No. of sites (%)	No. of isolates (%)	No. of isolates (%)
5[b]	40/195 (20.5)	6/53 (11.3)	3/64 (4.7)	4/30 (13.3)	5/40 (12.5)	10/20 (50.0)	16/30 (53.3)	8/28 (28.6)	8/35 (22.9)	4/26 (15.4)
22[b]	23/195 (11.8)	4/53 (7.5)	3/64 (4.7)	3/30 (10.0)	3/40 (7.5)	0/20 (0.0)	0/30 (0.0)	8/28 (28.6)	8/35 (22.9)	6/26 (23.1)
45	22/195 (11.3)	9/53 (17.0)	11/64 (17.2)	5/30 (16.6)	7/40 (17.5)	0/20 (0.0)	0/30 (0.0)	2/28 (7.1)	2/35 (5.7)	2/26 (7.7)
30	17/195 (8.7)	9/53 (17.0)	10/64 (15.6)	4/30 (13.3)	4/40 (10.0)	0/20 (0.0)	0/30 (0.0)	1/28 (3.6)	1/35 (2.9)	2/26 (7.7)
398	14/195 (7.2)	2/53 (3.8)	2/64 (3.1)	1/30 (3.3)	2/40 (5.0)	3/20 (15.0)	3/30 (10.0)	6/28 (21.4)	6/35 (17.1)	1/26 (3.8)
1	8/195 (4.1)	0/53 (0.0)	0/64 (0.0)	2/30 (6.6)	2/40 (5.0)	2/20 (10.0)	5/30 (16.6)	1/28 (3.6)	1/35 (2.9)	0/26 (0.0)
34	8/195 (4.1)	2/53 (3.8)	3/64 (4.7)	2/30 (6.6)	3/40 (7.5)	0/20 (0.0)	0/30 (0.0)	2/28 (7.1)	2/35 (5.7)	0/26 (0.0)
8	5/195 (2.6)	2/53 (3.8)	2/64 (3.1)	3/30 (10.0)	3/40 (7.5)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5031	5/195 (2.6)	1/53 (1.9)	1/64 (1.6)	1/30 (3.3)	2/40 (5.0)	1/20 (5.0)	2/30 (6.6)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
15	5/195 (2.6)	1/53 (1.9)	1/64 (1.6)	2/30 (6.6)	3/40 (7.5)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
5033	4/195 (2.1)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	3/28 (10.7)	3/35 (8.6)	0/26 (0.0)
6[b]	3/195 (1.5)	3/53 (5.7)	3/64 (4.7)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
508	3/195 (1.5)	3/53 (5.7)	3/64 (4.7)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
582	3/195 (1.5)	2/53 (3.8)	2/64 (3.1)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
1027	3/195 (1.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	1/28 (3.6)	1/35 (2.9)	2/26 (7.7)
20	2/195 (1.0)	0/53 (0.0)	0/64 (0.0)	1/30 (3.3)	1/40 (2.5)	1/20 (5.0)	1/30 (3.3)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
109	2/195 (1.0)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	2/26 (7.7)
5041	2/195 (1.0)	0/53 (0.0)	0/64 (0.0)	1/30 (3.3)	2/40 (5.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5043	2/195 (1.0)	0/53 (0.0)	0/64 (0.0)	1/30 (3.3)	1/40 (2.5)	1/20 (5.0)	1/30 (3.3)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
7	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
25	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)

Table 6.4 continued overleaf

Sequence type[a]	No. of isolates (%)	HCW		Patient		Environmental		Air		Clinical
		No. of HCWs (%)	No. of isolates (%)	No. of patients (%)	No. of isolates (%)	No. of areas (%)	No. of isolates (%)	No. of sites (%)	No. of isolates (%)	No. of isolates (%)
54	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	1/30 (3.3)	1/40 (2.5)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
59	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
96	1/195 (0.5)	1/53 (1.9)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
188	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	1/20 (5.0)	1/30 (3.3)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
320	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
672	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
789	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
972	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5029	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	1/20 (5.0)	1/30 (3.3)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5030	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	1/28 (3.6)	1/35 (2.9)	0/26 (0.0)
5034	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	1/30 (3.3)	1/40 (2.5)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5036	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
5039	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
5040[b]	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
5042	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
1160	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
1637	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
2233	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)
2383	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	1/28 (3.6)	1/35 (2.9)	0/26 (0.0)
2889	1/195 (0.5)	0/53 (0.0)	0/64 (0.0)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	1/26 (3.8)
3634	1/195 (0.5)	1/53 (1.9)	1/64 (1.6)	0/30 (0.0)	0/40 (0.0)	0/20 (0.0)	0/30 (0.0)	0/28 (0.0)	0/35 (0.0)	0/26 (0.0)

Footnotes overleaf

[a]Traditional MLST sequence types were assigned by analysing WGS contig sets using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany).

[b]Eighteen ST22 isolates were identified as ST22-MRSA-IV, five ST5 isolates were identified as ST5-MRSA-V, one ST6 isolate was identified as ST6-MRSA-IV and the ST5040 isolate was identified as ST5040-MRSA-IV. All other isolates were MSSA.

Abbreviations: HCW, healthcare worker.

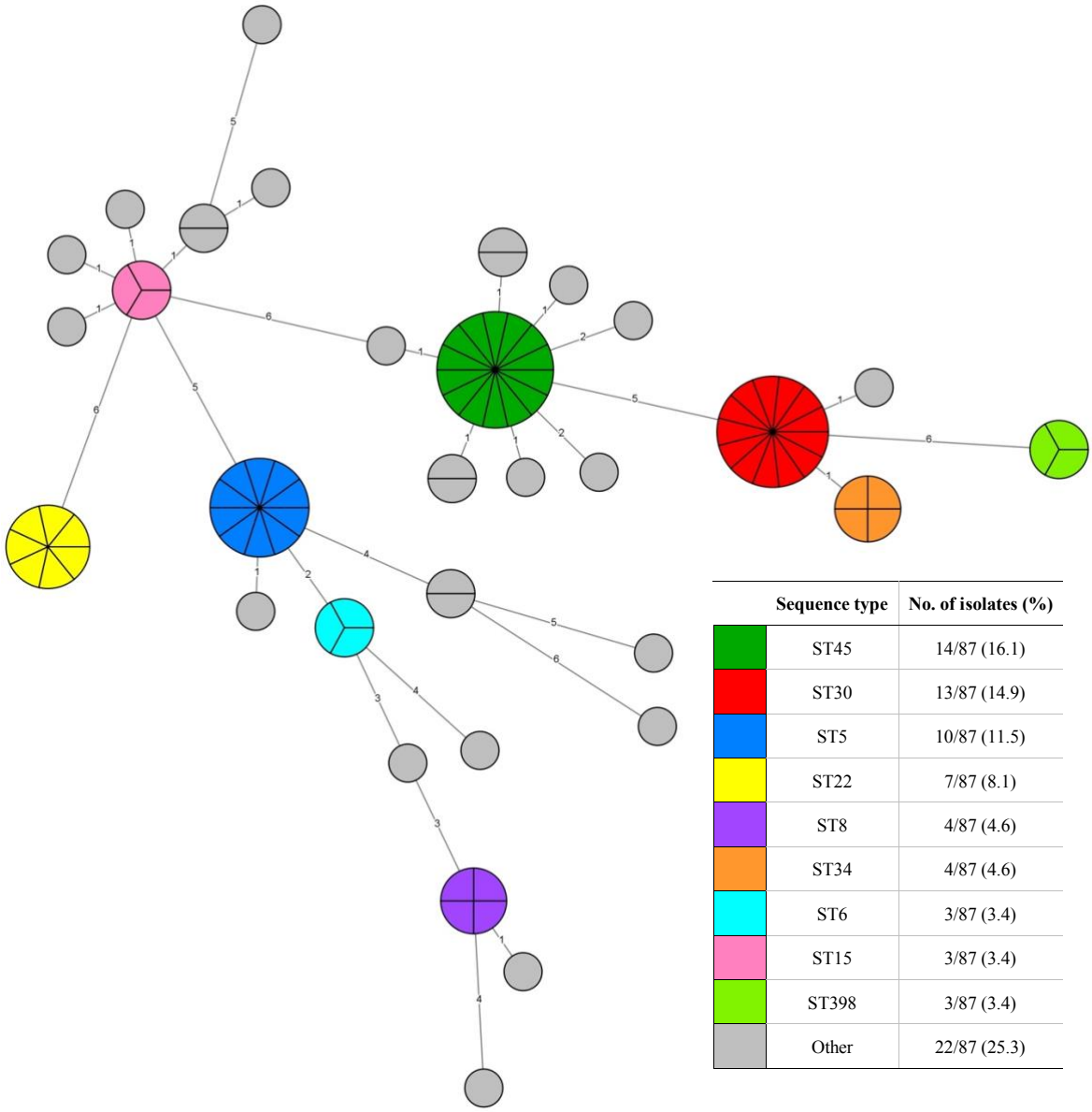
frequently identified lineage followed by ST22 (11.8%; 23/195) and ST45 (11.3%; 22/195). Correspondingly, *spa* type t548 (associated with ST5) was the most frequently identified *spa* type (12.3%; 24/195), followed by t032 (associated with ST22; 5.1%; 10/195) and t230 (associated with ST45; 4.6%; 9/195).

The population structure of *S. aureus* recovered from the participants is shown in Figure 6.2. A total of 31 different STs and 56 different *spa* types were identified among the 83 *S. aureus*-positive participants (Tables 6.2 and 6.4). Of the 21 participants who yielded both nasal and oral *S. aureus*, four individuals harboured two different STs. A total of 87 isolates were therefore investigated, including 80 MSSA and seven MRSA. All 31 STs were represented among the 80 MSSA isolates, while three of these STs were identified among the seven MRSA isolates. The most prevalent lineage was ST45 (16.1%; 14/87), followed by ST30 (14.9%; 13/87), ST5 (11.5%; 10/87) and ST22 (8.0%; 7/87), Fig. 6.2. Three of the ST22 isolates were identified as ST22-MRSA-IV and three of the ST5 isolates were identified as ST5-MRSA-V. The remaining MRSA isolate was identified as ST6-MRSA-IV. No STs were significantly more common among HCWs or patients. Indeed, HCWs and patients exhibited similar lineage prevalence trends and rates (Table 6.5). In HCWs, ST30 and ST45 (17.0%; 9/53) were the joint most prevalent lineages, followed by ST5 (11.3%; 6/53) and then ST22 (7.5%; 4/53). In patients, ST45 (16.6; 5/30%) was the most prevalent lineage, followed jointly by ST5 and ST30 (12.5%; 4/30), and then both ST8 and ST22 (10.0%; 3/30).

Eight different STs and 10 different *spa* types were identified among the 20 *S. aureus*-isolates recovered from near-patient areas (Tables 6.2 and 6.4). None of these isolates were MRSA. Twelve different STs and 22 different *spa* types were identified among the 28 *S. aureus* isolates recovered from air sampling sites (Tables 6.2 and 6.4). Eight different air sampling sites each yielded one ST22-MRSA-IV isolate and one air sampling site yielded an ST5-MRSA-V isolate. The 26 clinical isolates exhibited 14 different STs and 20 different *spa* types (Table 6.3; Table 6.5). Six clinical isolates were identified as ST22-MRSA-IV, while one was identified as ST5040-MRSA-IV (a ST8 SLV).

6.3.5 Phenotypic antimicrobial resistance trends

The prevalence of each resistance phenotype among the 195 isolates investigated is shown in Figure 6.3. Resistance to ampicillin (68.2%; 133/195) was particularly common. Erythromycin (28.7%; 56/195), fusidic acid (20.0%; 39/195) and ciprofloxacin (17.4%;



	Sequence type	No. of isolates (%)
	ST45	14/87 (16.1)
	ST30	13/87 (14.9)
	ST5	10/87 (11.5)
	ST22	7/87 (8.1)
	ST8	4/87 (4.6)
	ST34	4/87 (4.6)
	ST6	3/87 (3.4)
	ST15	3/87 (3.4)
	ST398	3/87 (3.4)
	Other	22/87 (25.3)

Figure 6.2 A minimum spanning tree based on the traditional multilocus sequence typing profiles of 87 *S. aureus* isolates recovered from HCWs and patients in a large Dublin hospital. Nasal and/or oral isolates were recovered from 53 HCWs and 30 patients on nine wards between May and October 2017. One isolate was included per participant, with the exception of the four instances in which an individual yielded isolates with two different STs. The "other" category includes 22 different STs recovered from 20 individuals. Branch labels indicate the number of allelic differences between nodes. Abbreviations: HCW, healthcare worker, ST, sequence type.

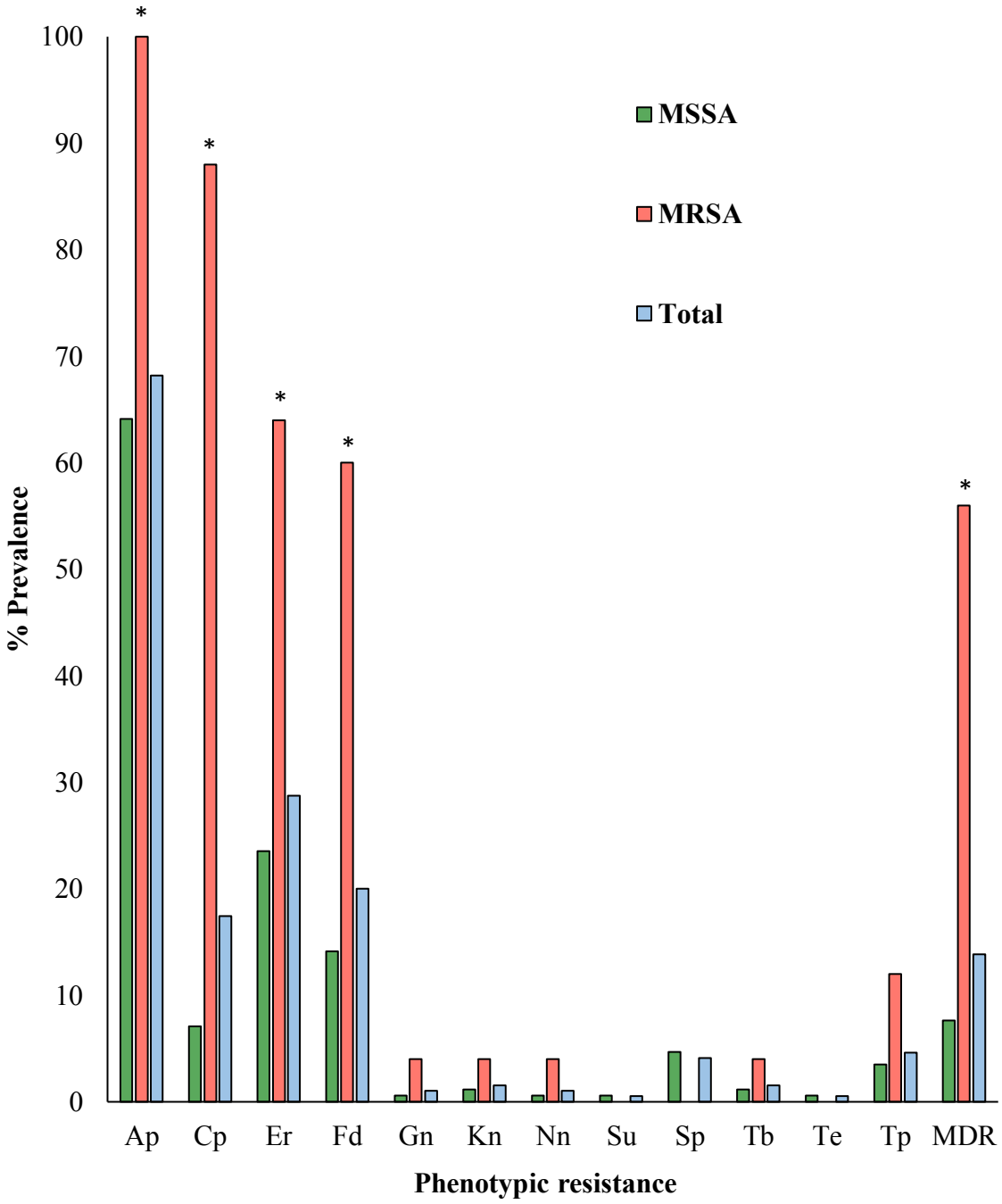


Figure 6.3 The resistance phenotypes detected among 170 MSSA and 25 MRSA isolates recovered on nine different wards of a large Dublin hospital in 2017. The 195 isolates investigated were recovered from 53 healthcare workers, 30 patients and their environment, 28 air sampling sites, 16 bloodstream infections and 10 skin and soft tissue infections. Statistically significant differences between MSSA and MRSA are indicated with an asterisk. Abbreviations: Ap, ampicillin; Cp, ciprofloxacin; Er, erythromycin; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Nm, neomycin; Su, sulphonamide; Sp, spectinomycin; Tb, tobramycin; Te, tetracycline; Tp, trimethoprim; MDR, multidrug-resistant.

34/195) resistance were also common. Spectinomycin resistance and tetracycline resistance were unique to MSSA, however, no resistance phenotypes were significantly more common in MSSA compared to MRSA. No resistance phenotypes were unique to MRSA, however, ampicillin, ciprofloxacin, erythromycin and fusidic acid resistance were all significantly more common in MRSA compared to MSSA ($p \leq 0.001$ in all instances). Gentamicin ($p = 0.114$), kanamycin ($p = 0.284$), neomycin ($p = 0.114$) and tobramycin ($p = 0.284$) resistance were also more common in MRSA compared to MSSA, however, these differences were not significant. Multidrug resistance was more prevalent in MRSA (56.0%; 14/25) than in MSSA (7.6%; 13/195), and this difference was significant ($p < 0.001$).

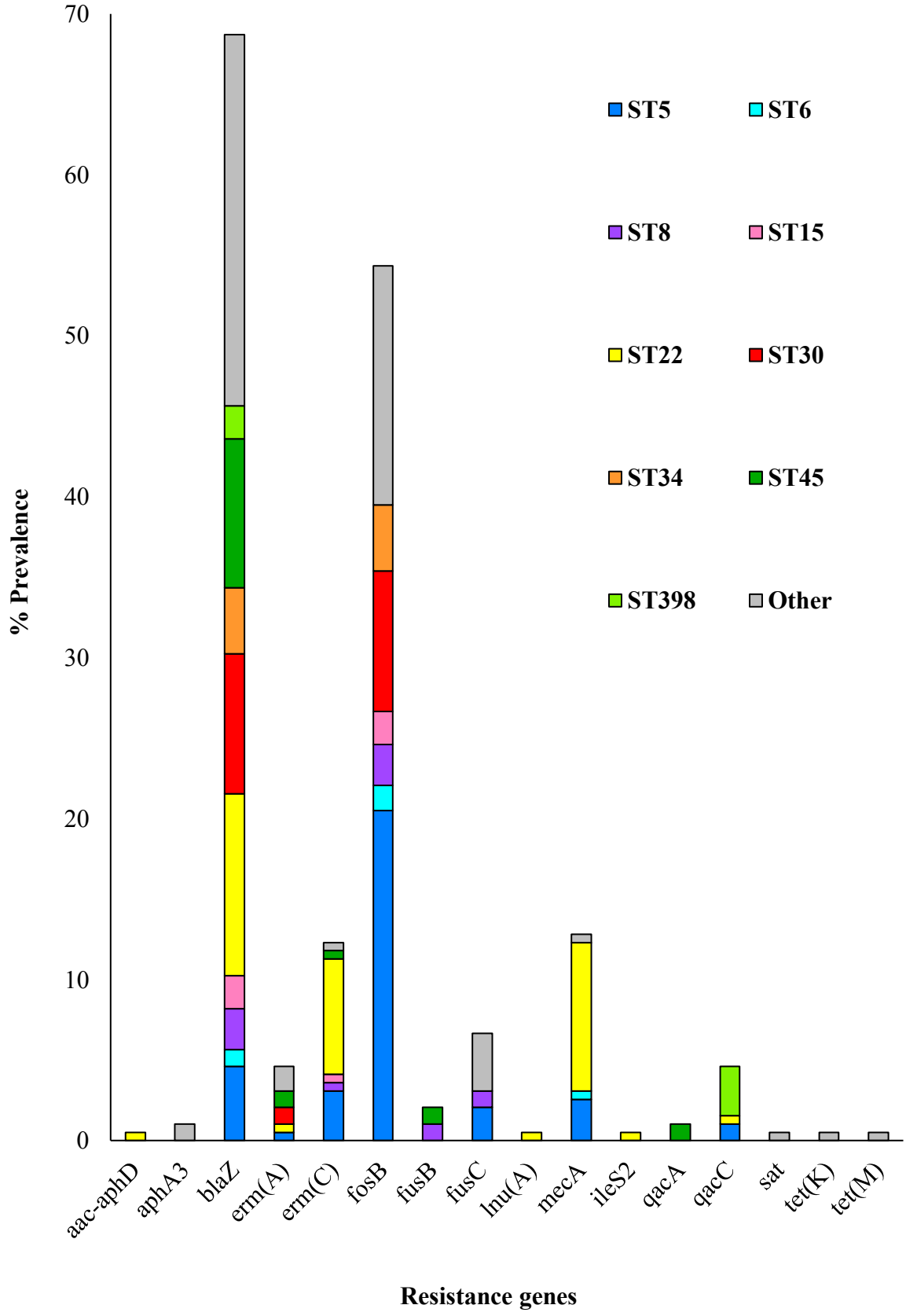
Several notable differences were observed between the resistance profiles of the HCW and patient isolates. Ampicillin resistance was significantly more common among HCW isolates (85.9%; 55/64) compared to patient isolates (65.0%; 26/40; $p = 0.012$). Ciprofloxacin resistance was also more common among HCW isolates (17.2%; 11/64) compared to patient isolates (5.0%; 2/40), however, this difference was not significant ($p = 0.067$). Fusidic acid resistance was more common among patient isolates (20.0%; 8/40) compared to HCW isolates (7.8%; 5/64), however, this difference was not significant ($p = 0.067$).

6.3.6 Resistance and virulence-associated genotypic trends

The prevalence rates of the major resistance and virulence-associated genes identified among the 195 isolates investigated are shown in Figure 6.4 (a) and (b), respectively.

The *blaZ* gene (68.7%; 134/195) was particularly prevalent and widespread among the different lineages (Fig. 6.4 [a]). The *fosB* (54.4%; 106/195) gene, which was detected in 7/9 STs, was also highly prevalent (Fig. 6.4 [a]). The *erm*(C) (12.3%; 24/195), *mecA* (12.8%; 25/195), *fusC* (6.6%; 13/195), *erm*(A) (4.6%; 9/195) and *qacC* (4.6%; 9/195) genes were relatively common, while the prevalence rates of the remaining resistance genes were low (0.5 - 2%; 1/195 - 4/195). The *fosB* ($p < 0.001$) gene was associated with ST5, while *aacA- aphD* ($p = 0.006$), *erm*(C) ($p < 0.001$), *lnu*(A) ($p = 0.006$) and *mecA* ($p < 0.001$) were associated with ST22, and *qacC* ($p < 0.001$) was associated with ST398. The *aacA-aphD* and *lnu*(A) genes were unique to ST22. The *qacA* gene was unique to ST5031, however, this association was not significant ($p = 0.800$).

A.


70
60
50
40
30
20
10
0
% Prevalence
ST5
ST6
ST8
ST15
ST22
ST30
ST34
ST45
ST398
Other
aac-aphD
aphA3
blaZ
erm(A)
erm(C)
fosB
fusB
fusC
lnu(A)
mecA
ileS2
qacA
qacC
sat
tet(K)
tet(M)
Resistance genes

B.

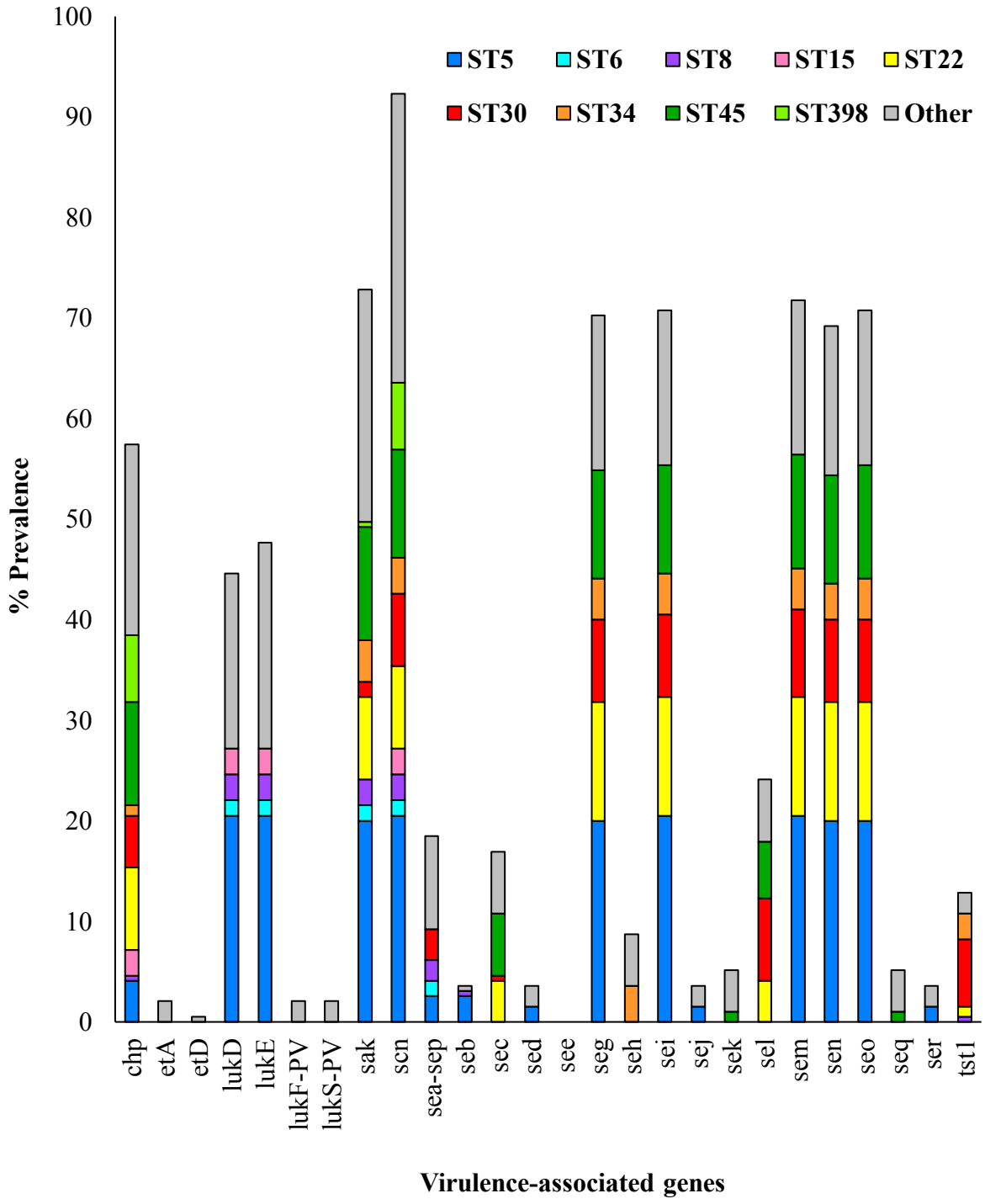


Figure 6.4 The prevalence of (A) resistance and (B) virulence-associated genes detected among 195 *S. aureus* isolates recovered on nine wards of a large Dublin hospital in 2017. The isolates investigated were recovered from 53 healthcare workers, 30 patients and their environment, 28 air sites, 16 bloodstream infections and 10 skin and soft tissue infections. The distribution of genes detected among the nine most prevalent sequence types identified among the study participants is indicated by the colour legend.

The virulence-associated genes were relatively well distributed among the different lineages (Fig. 6.4 [b]). The *scn* (92.3%; 180/195) gene was highly prevalent. The *sak* (72.8%; 142/195), *seg* (70.3%; 137/195), *sei* (70.8%; 138/195), *sem* (71.8%; 140/195), *sen* (69.2%; 135/195), *seo* (70.8%; 138/195), *chp* (57.4%; 112/195), *lukD* (44.6%; 87/195) and *lukE* (47.7%; 93/195) genes were relatively prevalent. The prevalence rates of the remaining virulence-associated genes were low to moderate (0.5-24.1%; 1/195-47/195). The *lukD*, *lukE*, *seg*, *sei*, *sem*, *sen* and *seo* genes were associated with ST5 ($p < 0.001$ in all instances). The *tst1* gene was associated with ST30 ($p < 0.001$).

6.3.7 Intra-hospital transmission

6.3.7.1 Development of data interpretation guidelines

In order to assess the relatedness of the 195 HCW, patient, environmental, air and clinical isolates and thus, identify recent transmission events, data interpretation guidelines were developed based in part on three different aspects of the wgMLST data. Firstly, assessment of the wgMLST dataset as a unit revealed that almost all (99.0%; 193/195) isolates could be divided into one of two groups. The first group consisted of 95/195 (48.7%) isolates, each of which differed from their closest relative by 0-26 alleles. The second group consisted of 98/195 (50.3%) isolates, each of which differed from their closest relative by 70-2108 alleles. The two remaining isolates differed from their closest relative at 45 and 46 wgMLST loci. Secondly, assessment of all instances ($n = 17$) in which two isolates (nasal and oral) of the same ST were recovered from a single participant revealed that a single strain could exhibit up to 17 allelic differences *in vivo*. This was concluded with the exception of a single instance in which a HCW simultaneously harboured two different ST22-MRSA-IV strains (125 allelic differences). Finally, upon assessment of all instances ($n = 8$) in which two or more isolates (bed frame, mattress and/or bedside locker) of the same ST were recovered in the same patient area, it was revealed that two strain variants recently transmitted from a common source (putatively the patient) could exhibit up to 21 allelic differences.

Considering these observations, combined with those detailed in previous studies (section 6.2.12), the following study-specific guidelines were established: (1) it is highly likely that two isolates exhibiting ≤21 allelic differences represent a recent transmission event, (2) it is possible that two isolates exhibiting 22-69 allelic differences represent a recent transmission event and all available epidemiological information should be assessed while considering this likelihood and (3) it is unlikely that two isolates exhibiting ≥70 allelic

differences represent a recent transmission event. Based on these guidelines, 16 potential transmission cases were identified involving a total of 71 (57 MSSA and 14 MRSA) isolates recovered from all nine wards. This included 13 HCW isolates from 11 different HCWs, 13 patient isolates from eight different patients, 22 environmental isolates from 14 different patient areas, 18 air isolates from 17 different air sampling sites and four SSTI isolates (Fig. 6.5).

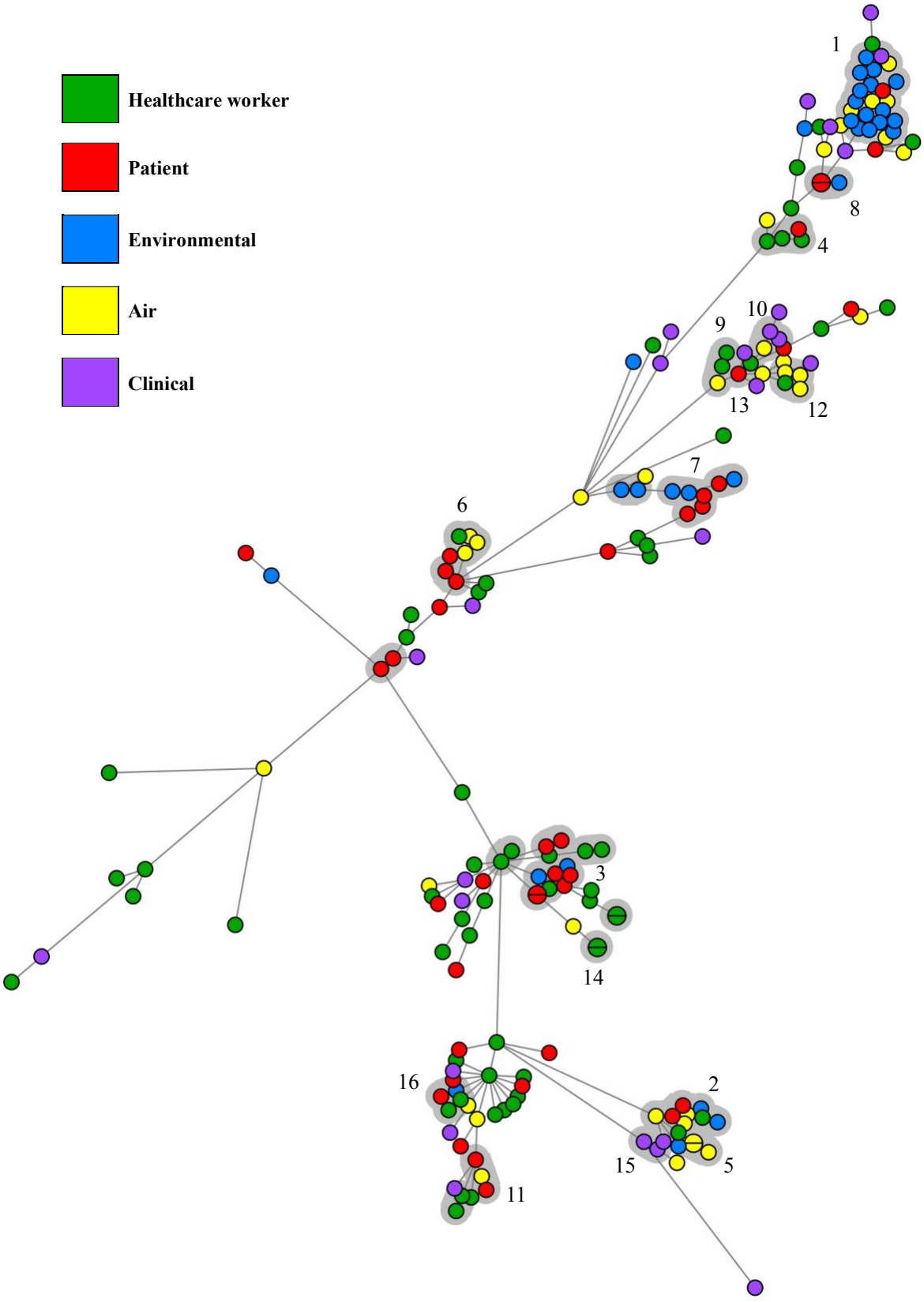
6.3.7.2 Case 1

A large cluster consisting of 22 ST5-MSSA isolates was identified among the 195 *S. aureus* isolates investigated. These isolates each differed from their closest relative by a maximum of 13 (average: 3.1) alleles and overall, exhibited 1-27 allelic differences. Almost all (21/22) of the isolates were recovered on ward A, while one isolate was recovered on ward C. These wards are on different floors of the study hospital. The ward A isolates were recovered throughout the ward (including the staff kitchen) over a three-week period. They included one patient isolate, five air isolates from five different sites and 15 environmental isolates from nine different patient areas. The ward A patient had not been admitted into the study hospital by the time the first case 1 isolate was recovered. The ward C isolate was a clinical isolate recovered from a patient who was not screened as part of this study. This isolate was recovered two weeks after the final ward A isolate, and differed from its closest relative by 5 alleles. It can be concluded from these data the a single ST5-MSSA strain was highly prevalent throughout ward A, and spread to at least one other ward. Furthermore, it is clear that the ward A patient acquired this strain during their hospital stay, possibly from the environment. While it appears likely that the ward C patient also acquired this strain during their hospital stay, this could not be definitively determined as their admission date was unavailable

6.3.7.3 Case 2

A second cluster was detected comprising eight ST398-MSSA isolates. These isolates each differed from their closest relative by a maximum of 13 (average: 4.7) alleles and overall, exhibited 1-19 allelic differences. All eight isolates were recovered on ward G, over a 16-day period. They included three air isolates from three different air sampling sites, two environmental isolates from two different patient areas, two patient isolates from a single patient and one HCW isolate. The patient isolates were recovered on day one of the 16 day period, while the HCW isolate was recovered from a nurse on day 15. Neither of the environmental isolates were associated with the patient. Six of the eight isolates were

A.


Healthcare worker
Patient
Environmental
Air
Clinical
1
8
4
10
9
13
12
7
6
3
14
16
2
15
5
11

B.

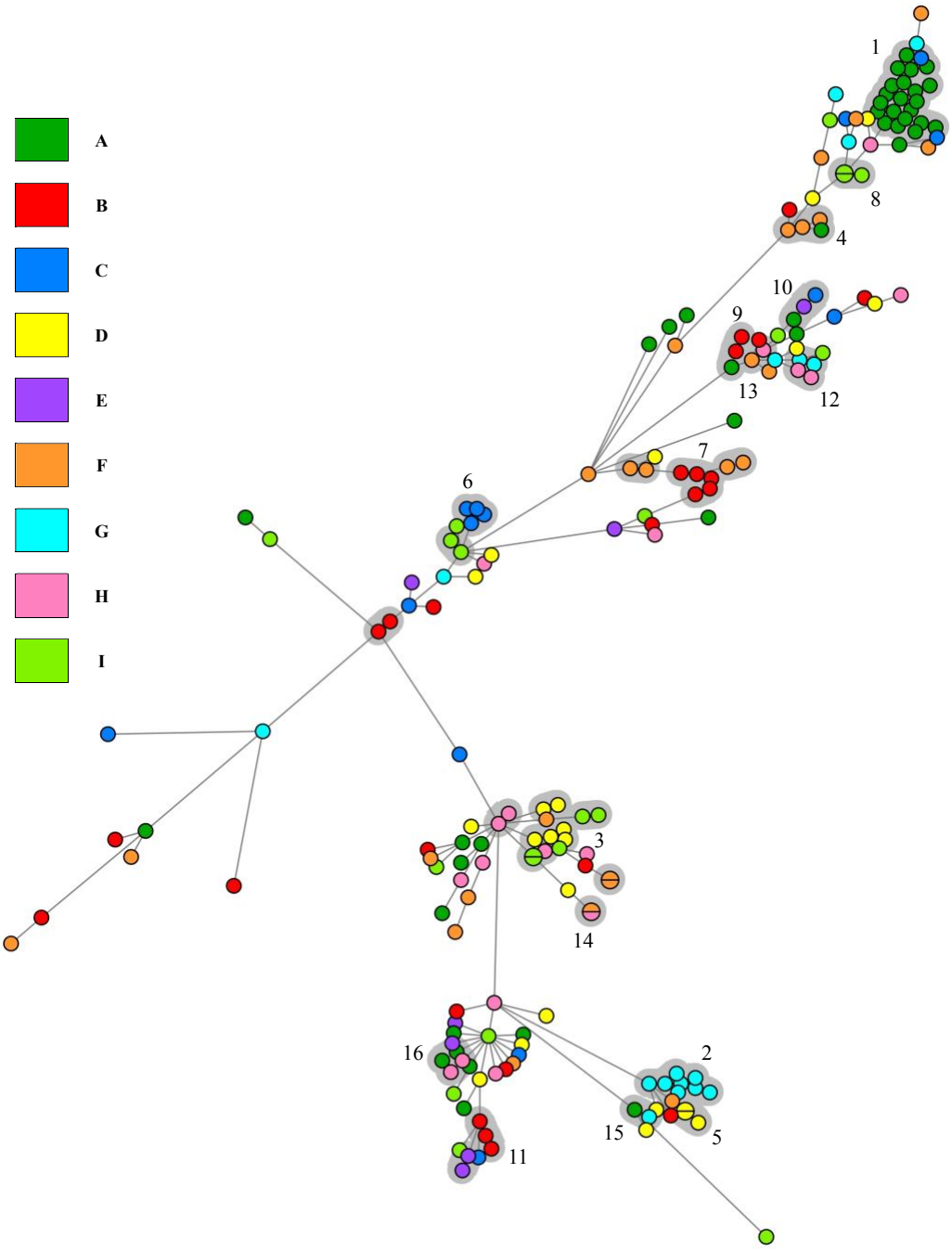


Figure 6.5 Minimum spanning trees based on the whole-genome multilocus sequence profiles of 195 *S. aureus* isolates recovered on nine wards of a large Dublin hospital in 2017. The isolates investigated were recovered from 53 healthcare workers, 30 patients and their environment, 28 air sites, 16 bloodstream infections and 10 skin and soft tissue infections. Closely related isolates are shaded in grey; those involved in the 16 cases described in section 6.3.7 are numbered accordingly; those remaining were recovered from single participants or environmental areas and thus, do not indicate recent transmission. The (A) isolate types and (B) wards on which the isolates were recovered, are indicated in the colour legends.

recovered in the same bay, while one air isolate was recovered in the neighbouring bay and another air isolate was recovered in the ward corridor. These data demonstrate that a single ST398-MSSA was prevalent in ward G, and predominantly focussed in a single bay. Although direct/indirect transmission has been identified between the nurse and patient, the original source of the strain remains unclear.

6.3.7.4 Case 3

A third cluster was identified consisting of five ST5031-MSSA isolates. These isolates each differed from their closest relative by a maximum of 10 (average: 3.8) alleles and overall, exhibited 1-12 allelic differences. All five isolates were recovered in ward D. They included one HCW isolate, two patient isolates from one patient and two environmental isolates from this patient's bed area. All isolates were recovered on the same day, with the exception of the HCW isolate, which was recovered one week later from a clerical worker. This suggests that the patient shed ST5031-MSSA into their immediate environment, however, it remains unclear whether the clerical worker directly/indirectly transmitted this strain to the patient or vice versa.

6.3.7.5 Case 4

A dispersed cluster of four ST5-MRSA-V isolates was identified. This included one patient isolate and three HCW isolates from two different HCWs. A healthcare assistant on ward A yielded the earliest recovered isolate, a nurse on ward F yielded the next two isolates 38 days later and a patient on ward H yielded the final isolate a further 21 days later. Wards F and H are on the same floor of the study hospital, while ward A is on a different floor. The two nurse isolates exhibited three allelic differences and were differentiated from the healthcare assistant isolate and patient isolate by 46 and 45 alleles, respectively. Overall, the cluster exhibited 3-51 allelic differences. The only other ST5-MRSA-V isolate identified during this study was recovered from the air on ward B, 55 days before the healthcare assistant isolate. This air isolate was differentiated from the case 16 isolates by 70 allelic differences. Together, these data suggest that while the three case 4 isolates may share a recent ancestral genotype, it appears unlikely that they represent recent intra-hospital transmission.

6.3.7.6 Case 5

A second ST398-MSSA cluster was detected, which was differentiated from the case 2 ST398-MSSA cluster by 165 alleles. This cluster comprised four ST398-MSSA isolates

which each differed from their closest relative by a maximum of 1 (average: 0.6) allele and overall, exhibited 0-2 allelic differences. This included one HCW isolate and three air isolates from three different air sampling sites. The HCW isolate was recovered from a healthcare assistant on ward F, while the three air isolates were recovered on ward D, 42 days later. Wards D and F are on different floors of the study hospital. Considering the particularly close relationship between the isolates, it appears likely that the healthcare assistant shed this strain into the air.

6.3.7.7 Case 6

A cluster consisting of four ST5033-MSSA isolates was also identified. These isolates each differed from their closest relative by a maximum of 17 (average: 12.6) alleles and overall, exhibited 8-19 allelic differences. All four isolates were recovered in ward C. They included one HCW isolate and three air isolates from three different air sampling sites. The three air isolates were recovered on the same day, while the HCW isolate was recovered two weeks later from a healthcare assistant. This suggests that the healthcare assistant may have been shedding ST5033 into the air. Alternatively, the healthcare assistant could have acquired this strain either from the air or its original source (i.e. another HCW or a patient).

6.3.7.8 Case 7

A small cluster was identified consisting of three ST22-MRSA-IV isolates. These isolates each differed from their closest relative by a maximum of 26 (average: 22) alleles and overall, exhibited 18-28 allelic differences. This relatively dispersed cluster included one air isolate recovered on ward E, a clinical isolate recovered in ward C and a second clinical isolate recovered on ward A. The ward E isolate was recovered first, the ward C isolate was recovered 41 days later and the ward A isolate was recovered a further 13 days later. Wards A and E are on the same floor of the study hospital, while ward C is located on a different level. The remaining 15 ST22-MRSA-IV isolates identified during this study were differentiated from the three case 7 isolates by 81-147 alleles. Considering the air isolate was recovered 41 days before the first clinical isolate, and that ST22-MRSA-IV is endemic in Irish hospitals, these data suggest that both the ward A patient and ward C patient acquired ST22-MRSA-IV during their respective hospital stays, possibly from the environment.

6.3.7.9 Case 8

ST1 MSSA was transmitted between a patient and the patient's own bed frame and bedside locker, in ward B. The patient occupied an open-ward bed at the time of sampling and yielded an oral isolate only. The three isolates each differed from their closest relative by nine alleles and overall, exhibited 9-18 allelic differences. Considering ST1 MSSA was not prevalent in this study (it was recovered only from two other patients and their respective environments, and one air sampling site), it is likely that the strain was transmitted from the patient to the environment.

6.3.7.10 Case 9

ST5 MSSA was transmitted between a patient in ward I and the patient's own bed frame. The patient occupied a single room at the time of sampling. Both nasal and oral isolates, which exhibited zero allelic differences, were recovered from the patient. The patient isolates differed from the environmental isolate by two alleles. This suggests the strain was transmitted from the patient to the environment, however, it remains possible that the patient acquired it from their bed frame.

6.3.7.11 Case 10

ST22-MRSA-IV was transmitted between a nurse in ward B and the air of the staff kitchen in ward A. The nurse yielded both nasal and oral ST22-MRSA-IV which exhibited 4 allelic differences. The three isolates each differed from their closest relative by a maximum of 11 (average: 7.5) alleles and overall, exhibited 4-12 allelic differences. The air isolate was recovered 64 days after the HCW isolates. Wards A and B are beside each other on the same floor of the study hospital. The dynamics of this transmission case remain unclear. While the nurse may have been shedding this strain, it is possible that (i) the nurse transmitted the strain to a different HCW/patient who subsequently shed it into the air of ward A or, (ii) the strain was shed by a different HCW/patient, who had previously transmitted it to the nurse.

6.3.7.12 Case 11

ST34 MSSA was transmitted between a patient and the air in ward B. The patient yielded both nasal and oral isolates, which exhibited 12 allelic differences, and occupied an open-ward bed at the time of sampling. The air isolate was recovered one week after the patient isolates in the same bay. It differed from the oral isolate by 1 allele. Considering the

particularly close relationship between the air and oral isolates, it appears likely that the patient shed this strain into the air.

6.3.7.13 Case 12

ST22-MRSA-IV was transmitted between a nurse and the air in ward H. The nurse yielded nasal *S. aureus* only. The air isolate was recovered 43 days after the HCW isolate. The two isolates exhibited 10 allelic differences. This case mirrors case 10, described above. While the nurse may have been shedding this strain, it is possible that (i) the nurse transmitted the strain to a different HCW/patient who subsequently shed it into the air or, (ii) the strain was shed by a different HCW/patient, who had previously transmitted it to the nurse.

6.3.7.14 Case 13

ST22-MRSA-IV was transmitted between a nurse on ward H and a patient on ward F, both of whom yielded oral isolates only. The HCW isolate was recovered six days after the patient isolate. The two isolates exhibited four allelic differences. Wards H and F are on the same floor of the study hospital. It remains unclear whether the HCW transmitted this strain to the patient or vice versa.

6.3.7.15 Case 14

ST45 MSSA was transmitted between a healthcare assistant in ward F and a nurse in ward H, both of whom yielded oral isolates only. These two isolates were recovered on the same day and exhibited zero allelic differences. Wards F and H are on the same floor of the study hospital. It is likely that transmission occurred between these HCWs particularly recently. However, the direction of transmission could not be determined.

6.3.7.16 Case 15

ST1027 MSSA was transmitted between and caused infections in two patients. One of the isolates was recovered from a patient infection in ward A, while the second isolate was recovered from a patient infection in ward G, the next day. Wards A and G are on different floors of the study hospital. Neither of these patients were screened as part of the present study. Their isolates exhibited seven allelic differences. This suggests that at least one of these patients acquired this strain during their hospital stay.

6.3.7.17 Case 16

ST5043 MSSA was transmitted between a patient and the patient's own bed frame in ward A. The patient occupied an open-ward bed at the time of sampling and yielded a nasal isolate only. The two isolates exhibited 17 allelic differences. Considering ST5043 is an unusual ST (it was not detected among any other isolates investigated), it is highly likely that the patient transmitted the strain to their bed frame.

6.3.7.18 Summary of transmission cases

- Fifteen likely transmission cases were identified, involving 68/195 (34.9%) isolates. Twelve of these cases involved MSSA, while three involved MRSA.
- A total of 3/30 *S. aureus*-positive patients harboured a strain closely related to that of a HCW, and 3/53 *S. aureus*-positive HCWs harboured a strain closely related to that of a patient. In all three corresponding cases (2, 3 and 13), one of which involved MRSA, the direction of transmission could not be determined.
- One likely case (5) and three possible cases (6, 10 and 12) were identified in which a HCW shed *S. aureus* into the air.
- One case (14) was identified in which MRSA was transmitted between two different HCWs.
- Four patients (case 1; ward A patient, case 7, both patients; case 15, either patient) were identified who likely acquired *S. aureus* during their respective hospital stays. The air and/or environment was identified as a possible source of the acquired strains for three of these patients.
- Two patients (case 1; ward C patient; case 15, either patient) were identified who may have acquired *S. aureus* during their respective hospital stays. The air and/or environment was identified as a possible source of the acquired strain for one of these patients.
- Two patients (cases 8 and 16) were identified who likely transmitted *S. aureus* to their immediate surroundings, and one patient (case 9) was identified who may have transmitted *S. aureus* to their immediate surroundings.
- One patient (case 11) was identified who likely shed *S. aureus* into the air.

6.4 Discussion

This study investigated the prevalence and transmission of *S. aureus* in a large Irish hospital where MRSA is endemic. Following the sampling of 149 HCWs, 132 patients and their immediate environment, and 135 air sampling sites over a six-month period, a total of 15 transmission cases were identified involving ~35% of all isolates investigated. Interestingly, 12 of these cases involved MSSA, which are not typically the focal point of IPC strategies. While the environment was identified as a significant reservoir for MRSA/MSSA, only three instances of transmission between a patient and HCW were identified. Therefore, although previous studies strongly indicate that HCWs constitute a significant source of MRSA/MSSA in patients during outbreaks, this investigation indicates that *S. aureus* is rarely transmitted between HCWs and patients in non-outbreak scenarios. The present study also provided valuable insight into the population structure of MSSA in Ireland, of which little is currently known. Among a highly diverse set of MSSA lineages, ST45, ST30 and ST5 were particularly prevalent in both HCWs and patients. Finally, this study has highlighted clinically relevant phenotypic and genotypic antimicrobial resistance trends among *S. aureus* in Ireland.

As detailed in Chapter 1, section 1.1.1, cross sectional studies report *S. aureus* carriage rates of approximately 30% among the general population in the UK and USA (Gamblin et al., 2013; Mainous et al., 2006). While the overall prevalence of *S. aureus* in the present study (29.5%) corresponds well with this estimation, the rate of carriage was lower than anticipated among patients (22.7%) and higher than anticipated among HCWs (35.6%). Although this difference cannot be definitively explained, it is likely attributable in part to the recent use of antibiotics by 73.5% of the patient participants versus 12.1% of the staff participants. Regarding MRSA specifically, a study carried out during 2010/2011 in the same hospital as the present study, reported that MRSA was recovered from 4.3% (30/706) of patients (Creamer et al., 2014). This rate is higher than that identified in the present study (1.5%), suggesting that MRSA carriage rates may have decreased in the study hospital between 2010 and 2017. This is likely a reflection of the aforementioned trends which indicate that hospital-acquired MRSA infection rates are decreasing in Europe, while hospital-acquired MSSA infection rates are increasing (EARS-Net, 2009, 2017b). Interestingly, however, the rate of MRSA carriage among HCWs (3.4%) corresponds with previous reports which indicate that 2.3-4.8% of HCWs in Ireland harboured MRSA between 2003 and 2010 (Dulon et al., 2014; Edmundson et al., 2011).

The identification of just three instances in which *S. aureus* was transmitted between a patient and HCW, in an unknown direction, suggests that patients rarely acquire *S. aureus* from HCWs in non-outbreak settings. Interestingly, however, one likely and three possible instances of a HCW shedding *S. aureus* into the air were also identified and thus, it remains possible that these strains were acquired by patients who did not participate in the study. Nonetheless, all nine wards investigated were characterised by a constant influx of new genotypes, rather than the transmission of a limited number of strains (Fig. 6.5 [b]). These findings are in agreement with those detailed in a recent WGS-based transmission study set in two high-dependency units in the UK, where MRSA is also endemic (Price et al., 2017). In this study, 198 HCWs, 1854 patients and 40 environmental sites were routinely sampled over a 14-month period. Despite thorough sampling and the increased level of direct patient contact associated with high-dependency units, only seven instances were identified in which *S. aureus* was transmitted from a HCW to a patient (Price et al., 2017). Together, these studies suggest that the routine screening of HCWs for MSSA/MRSA would not significantly decrease the number of patients who acquire *S. aureus* during their hospital stay.

The identification of at least three patients who may have acquired *S. aureus* from the air and/or the environment is consistent with previous investigations that have identified the hospital environment as a significant reservoir for *S. aureus* (Creamer et al., 2014; Price et al., 2017; Wang and Ruan, 2017). Regarding MRSA specifically, aerial/environmental contamination rates have apparently decreased in the study hospital over the past seven years. During the aforementioned 2010/2011 investigation in the study hospital, MRSA was detected among 14.4% (191/32) of all air samples, while only 6% of the air sites sampled in the present investigation yielded MRSA (Creamer et al., 2014). Furthermore, in 2010/2011, MRSA was recovered from 1.9%, 3.7% and 6.2% of bed frames, bedside lockers and mattresses, respectively, while 0% of each these areas yielded MRSA in the present study (Creamer et al., 2014). While the reduced rate of aerial contamination may be fully attributable to the decreased prevalence of MRSA in Irish hospitals, it is possible that the perceived elimination of environmental contamination is partially attributable to variances in methodology. A recent study determined that broth enrichment of environmental swabs prior to subculturing onto chromogenic agar can increase the proportion of *S. aureus*-positive samples by 46% (Mernelius et al., 2013). In the 2010/2011 study, all environmental swabs were enriched in tryptone soy broth with 6% (w/v) NaCl, however, this was not performed during the present investigation.

The extensive diversity of the MSSA population in Ireland was highlighted by the identification of 31 different MSSA STs among 83 *S. aureus*-positive individuals. Few studies have investigated the molecular epidemiology of MSSA in Ireland, the two most comprehensive of which, have focussed exclusively on MSSA recovered from invasive infections (Grundmann et al., 2010, 2014). These linked structured surveys used *spa* typing to investigate the molecular epidemiology of *S. aureus* in Europe in 2006, and again in 2011. In 2006, ST30 (11.8%) was identified as the most prevalent MSSA lineage in Ireland, while ST5 (4.8%) predominated throughout Europe, followed by ST15 (4.6%) and ST45 (4.4%) (Grundmann et al., 2010). In 2011, ST7 (5.3%) was identified as the most prevalent MSSA lineage in Europe, followed by ST15 (4.7%) and ST5 (4.6%) (Grundmann et al., 2014). The predominant MSSA lineage in Ireland was not specified. These invasive *S. aureus* trends are consistent with the identification of ST45, ST30 and ST5 as the predominant carriage lineages in the present investigation. Indeed, previous studies have demonstrated that invasive infections are usually caused by endogenous strains (von Eiff et al., 2001; Wertheim et al., 2004). As only seven individuals yielded MRSA, their isolates could not be assessed as a microcosm of the MRSA population in Ireland. Unsurprisingly, however, the majority of MRSA isolates were characterised as either ST22-MRSA-IV, the predominant clone in Irish hospitals, or ST5-MRSA-IV, the fourth most prevalent clone in Irish hospitals in 2016 (Irish NMRSARL, 2016). Interestingly, ST6-MRSA-IV, which is not typically associated with Ireland, was also detected during this study. Strains matching the description of this clone have previously been identified in Australia and the UAE (Monecke et al., 2011).

Phenotypic antimicrobial resistance testing demonstrated that *S. aureus* in Ireland often exhibit resistance to penicillin, erythromycin and ciprofloxacin, all of which are frequently consumed in both hospitals and the community, in Ireland (Irish HPSC, 2017). Resistance to fusidic acid, which is often used in the community throughout Europe (Dobie and Gray, 2004; Mason and Howard, 2004) was also highly prevalent. Notably, however, fusidic acid usage is not monitored as part of the European antimicrobial consumption surveillance system. Similarly, genotypic analysis revealed that *fosB* is highly prevalent among *S. aureus* in Ireland, however fosfomycin consumption is not systematically monitored in Europe.

Several limitations of this investigation merit consideration. Firstly, as participation in this study was voluntary, the sample size was limited and some HCW roles were poorly

represented. This may have affected attempts to determine whether certain employment categories are associated with increased *S. aureus*/MRSA carriage rates. Indeed, previous research indicates that MRSA carriage is more common among nurses/healthcare assistants compared to other healthcare professionals (Dulon et al., 2014; Verwer et al., 2012), however, this could not be definitively concluded during the present investigation. Secondly, as HCWs were sampled only once during the study period, the potential involvement of non-persistent carriers in transmission cases may have gone undetected. Finally, the constraints associated with analysing one isolate per patient were highlighted by the recovery of two unrelated strains from five of the 21 participants for whom two isolates (nasal and oral) were investigated. Unfortunately, this obstacle is common among WGS-based studies, as costs are often an important factor for consideration.

The present investigation constitutes the first phase of a three-phase study. Phases two and three will follow the same structure as phase one, with the intention of recruiting as many HCWs as possible who were sampled during the previous phase(s). This will increase the size of the sample group and thus, the certainty with which conclusions may be drawn. Furthermore, these additional phases will facilitate the identification of persistent versus non-persistent HCW carriers. Finally, as many HCWs, environmental areas and air sampling sites yielded *S. aureus* during phase one, it may be possible to definitively determine the direction of transmission for a greater proportion of transmission cases during phases two and three.

In conclusion, this investigation has indicated that HCWs rarely transmit *S. aureus* to patients in non-outbreak settings, suggesting that the routine screening of HCWs for MSSA/MRSA would not be practical in MRSA-endemic settings. Furthermore, this study has highlighted the genotypic diversity of MSSA in Ireland, while identifying ST45, ST30 and ST5 as particularly prevalent. Finally, this investigation has indicated that *S. aureus* in Ireland are often resistant to penicillin, macrolides, ciprofloxacin, fusidic acid and fosfomycin.

Chapter 7

General Discussion

7.1 Preventing CA-MRSA transmission in Irish hospitals

As highlighted by the studies described in Chapters 3, 4 and 5 of this work, CA-MRSA is becoming increasingly prevalent in Irish healthcare settings. This is reflected by similar findings in many other parts of the world (David and Daum, 2010; Otter and French, 2010). Monitoring and ultimately controlling the spread of these clones is of utmost importance. Several studies have reported the displacement of previously predominant HA-MRSA clones by CA-MRSA clones. For example, in India, the MDR CA and PVL-positive CC1-ST772-MRSA-V clone has displaced the previously predominant HA ST239-MRSA-III clone (Chuang and Huang, 2013; Monecke et al., 2011). In the USA, the CA PVL-positive CC8-ST8-MRSA-IV clone, USA300, now constitutes the leading cause of MRSA nosocomial infections, having displaced the previously predominant HA CC5-ST5-MRSA-II clone, USA100 (David et al., 2014). The two major sources of CA-MRSA in hospitals are patients and HCWs.

The current Irish national MRSA IPC guidelines consider risk factors associated with HA-MRSA carriage only during targeted patient screening (Irish Department of Health, 2013). Considering the global emergence of CA-MRSA, this approach has become somewhat outdated. Implementing universal patient screening (i.e. screening all patients on admission into hospital) is an alternative option, which has proven to be successful in European countries with low MRSA prevalence rates, such as The Netherlands (Souverein et al., 2016). However, current evidence indicates that this approach may not be suitable for regions in which MRSA in endemic, such as Ireland (Irish Department of Health, 2013). In 2011, a study was undertaken in a large Dublin hospital in which 892 patients underwent both universal and targeted screening. It was found that 8% of at-risk patients and 1% of non-risk patients were MRSA positive (i.e. an additional four patients were detected that would not normally have been screened), however, the programme was associated with significantly increased costs (approximately 33%) (Irish Department of Health, 2013). Furthermore, when universal screening was implemented in a hospital in the UK, where MRSA is also endemic, results of a one-year programme showed that short hospital stays often resulted in failure to complete decolonisation regimes (Irish Department of Health, 2013). It may therefore be more pragmatic to continue targeted patient screening in Ireland, however, the risk factors considered should perhaps be extended to include those associated with CA-MRSA.

Extensive studies regarding CA-MRSA risk factors are lacking in Ireland and most of mainland Europe. In the absence of such data, CA-MRSA risk factors are often inferred from studies from the USA. As detailed in Chapter 1, section 1.8.2, these studies have linked CA-MRSA to participation in contact sports, living in overcrowded conditions such as prisons, and having a low socioeconomic status, among other factors (David et al., 2011). Interestingly, these factors were not associated with CA-MRSA in Switzerland, where being of non-European origin was linked to CA-MRSA colonisation (Longtin et al., 2009). Furthermore, being of non-European origin was not associated with CA-MRSA in The Netherlands, where poultry consumption and cattle density were linked to CA-MRSA colonisation (van Rijen et al., 2013). Together, these data suggest that CA-MRSA risk factors may vary in different geographical regions, highlighting the importance of studies that investigate CA-MRSA risk factors in Ireland, specifically. While a previous study (using PVL as a marker for CA-MRSA), identified a link between CA-MRSA carriage/infection in Ireland and international travel, young people and shared housing (Shore et al., 2014), additional studies are needed to investigate this issue further.

Healthcare workers have repeatedly been implicated in CA-MRSA outbreaks (Brennan et al., 2012; Nagao et al., 2010; Ramsing et al., 2013), as was the case in the research described in Chapters 3 and 5 of this work, suggesting they too constitute an important source of CA-MRSA in hospitals. Current Irish national MRSA IPC guidelines recommend HCW screening only following the identification of an infection cluster (Irish Department of Health, 2013). However, as suggested by the findings of the present study, this approach may not be sufficient to prevent onwards transmission of CA-MRSA by colonised HCWs. While the routine screening of HCWs for MRSA does not appear practical, as indicated in Chapter 6, it is possible that smaller changes to the current guidelines could significantly reduce the frequency with which HCWs introduce CA-MRSA into nosocomial settings. For example, in Irish hospitals, the identification of an infection cluster among patients (and the subsequent administration of antibiotics) is currently the threshold that must be met in order to trigger HCW screening. Perhaps, however, the implementation of HCW screening upon identification of a colonisation cluster among patients would also be appropriate. Furthermore, upon detection of an infection cluster, HCW screening is not currently mandatory and the degree to which it is implemented in individual hospitals is unknown. It is conceivable that some HCWs may be reluctant to participate in MRSA screening due to fear of the consequences, such as removal from clinical duties until decolonised. It may therefore be beneficial to provide

HCWs with further education, support and reassurance regarding this issue. Finally, as international travel was identified as a likely contributing factor to the outbreaks described in both Chapters 3 and 5 of this work, HCW screening following a period of time abroad may also be appropriate.

Finally, in maternity hospitals in particular, both mothers and their partners have also been identified as signicant sources of CA-MRSA among neonates (Harris et al., 2013; Menchini et al., 2013; Touveneau et al., 2006). Despite these findings, current Irish national guidelines recommend screening mothers only for MRSA, and not their partners (Irish Department of Health, 2013). Studies investigating the potential efficacy of including partners as screening targets in maternity hospitals may therefore be warranted.

7.2 Antimicrobial resistance

The European CC1-MRSA-IV clone defined in Chapter 4 of this study exhibited resistance to aminoglycosides, MLS_B compounds and tetracyclines, in addition to β-lactams. Among the 195 *S. aureus* isolates investigated in Chapter 6, resistance to fluoroquinolones, MLS_B compounds and fusidic acid (in addition to β-lactams) was particularly common, and 56.0% of all MRSA and 7.6% of all MSSA isolates were identified as MDR. Furthermore, all the CC88-MRSA isolates investigated in Chapter 5 exhibited resistance to at least one non-β-lactam antibiotic class, mostly frequently MLS_B compounds. These data reflect the ongoing global antimicrobial resistance crisis, which the World Health Organisation has identified as one of the greatest threats to global health (World Health Organisation, 2014). Infections caused by resistant bacteria such as MRSA, vancomycin-resistant enterococci or carbapenem-resistant *Enterobacteriaceae* (CRE) are associated with increased morbidity and mortality. Furthermore, the effects of antimicrobial resistance constitute a significant economic burden on the healthcare systems worldwide, costing the USA healthcare system alone, an estimated $21-34 billion per year (World Health Organisation, 2014). The routine application of WGS in research has provided extensive data relevant to both the identification of new antibiotic targets and the evolutionary dynamics responsible for the development of antimicrobial resistance (Donkor, 2013; Gillings et al., 2019). The emergence of methicillin resistance in staphylococci is particularly well studied, and could provide valuable insight into the evolution of other resistance genotypes.

Evidence indicates that the continuous exposure of staphylococci to β-lactams in soils (where they co-exist with penicillin-producing fungi), farm animal food additives, and during the treatment of infections, led to the widespread emergence of β-lactam and methicillin-resistant staphylococci (Miragaia, 2018; USA Research Council, 1980; Westh et al., 2004). Sequence-based studies indicate that *mecA* evolved from a native staphylococcal protein (PBPD) in *S. sciuri,* a staphylococcal species prevalent in animals (Rolo et al., 2017b). It is likely that *mecA* was then transferred to other animal-associated staphylococcal species (*Staphylococcus fleuretti* and *Staphylococcus vitulinus*), where it acquired regulatory genes involved in the control of its expression (*mecRI*/*mecI*) (Rolo et al., 2017b; Tsubakishita et al., 2010). The resulting *mecA* complex was then acquired by *S. sciuri*, and integrated into a SCC element, which had evolved independently at *orfX* (Rolo et al., 2017a). Other animal-associated staphylococcal species subsequently acquired SCC*mec* and eventually, SCC*mec* disseminated among staphylococci prevalent in both animals and humans, such as *S. epidermidis* and *S. aureus* (Miragaia, 2018). Importantly, this all occurred before the introduction of methicillin into clinical practice (Harkins et al., 2017).

These findings provide three general lessons regarding antimicrobial resistance. Firstly, as demonstrated by the origin SCC*mec* in animals, this evidence emphasises the importance of the livestock industry in the development of antimicrobial resistance. Since the 1950s, antibiotics have commonly been added to the feed of healthy farm animals in order to prevent infections and promote growth (USA Research Council, 1980). In addition to encouraging selection for resistance *in vivo*, this practice can also contribute to low-level antibiotic environmental contamination, which has recently been linked to the emergence of CA-MRSA (Gustave et al., 2018). While banned in Europe and the USA in 2006 and 2017, respectively, this practice continues in many other parts of the world (Cogliani et al., 2011; Cuong et al., 2016; Zhang et al., 2019). Secondly, as demonstrated by the dissemination of SCC*mec* among human staphylococcal strains, these findings highlight the importance of antimicrobial stewardship in human medicine. Interestingly, it has recently been suggested that the duration for antibiotic courses should be reduced (Llewelyn, 2017). A consequence of this welcome discussion, however, has been the delivery of mixed messages to the general public, via mainstream media (Nature Editorial, 2017). This may have unintended consequences; for example, patients may gradually reduce their dose (rather than the duration of their course) when they start to feel better, likely selecting for resistance (Nature Editorial, 2017). It is therefore essential that this

matter is investigated further to ensure both the development of safe and responsible prescription guidelines, and the delivery of clear messages to the general public. Thirdly, the evolution of MRSA prior to the introduction of methicillin into clinical practice demonstrates that unidentified adaptations in bacterial populations can render novel antibiotics ineffective sooner than anticipated (Harkins et al., 2017).

7.3 The application of WGS-based typing methods to MRSA

All four research components of the present study utilised WGS-based typing methods to track the spread of MRSA within hospitals, between hospitals or on an international scale. The techniques used varied in each chapter, depending on the purpose of the investigation and the resources available at the time of the analyses. They included both SNV-based and loci-based approaches.

In Chapter 3, a cgMLST-based MST was generated and a major cluster was identified, including one tight sub-cluster (sub-cluster I) and one dispersed sub-cluster (sub-cluster II). A second cgMLST-based MST and a SNV matrix were then generated using the cluster isolates only. Upon identification of the major cluster, however, it may have been more informative to examine the sub-cluster I isolates independently. As these isolates were very closely related, consideration of the entire genome (either via wgMLST or SNV analysis using a sub-cluster I reference) may have generated a more accurate phylogeny with which to infer the transmission dynamics of the outbreak. This retrospective observation is merely a reflection of WGS becoming more widely used during the course of this study, and the advantages and disadvantages associated with different analysis methods becoming better appreciated and understood among microbiologists (Carriço et al., 2018; Humphreys and Coleman, 2019; Schürch et al., 2018).

In Chapter 4, the application of cgSNV analysis permitted the nucleotide-level comparison of both closely and distantly related isolates, without the use of a reference genome. This reduced the chances of read mismapping, which can be caused by the use of an inappropriate reference (i.e. one which is not closely related to all isolates in the analysis). The disadvantage of this method, however, was the exclusion of intergenic regions, which possibly reduced resolution. In addition to cgSNV analysis and standard SNV analysis, a third SNV-based method exists, whereby the reads of the query genomes are mapped against a reference, and all accessory genes are removed prior to SNV calling (Aanensen et al., 2016; Gordon et al., 2017; Holden et al., 2013; Köser et al., 2012). The application of

this technique currently requires significant bioinformatic expertise. No studies published to date have thoroughly investigated the advantages and disadvantages associated with each of these approaches, all three of which are generally referred at as SNV/SNP analysis, in the literature. Such studies could facilitate and enhance the finetuning of *S. aureus* transmission investigations.

In Chapter 5, wgMLST was used to investigate the transmission dynamics of the outbreak strain, while cgMLST was used to compare more distantly related isolates. The identification of a signature rare deletion among the outbreak isolates indicated that the application of wgMLST or SNV analysis, rather than cgMLST, was more appropriate for the particular dataset. This highlighted the importance of considering various different approaches during WGS data analysis. Ideally, an analysis pipeline should trial numerous different methods before selecting the most appropriate technique(s) for detailed data interpterion. Similarly, the generation of multiple phylogenetic trees is advisable during data analysis. In the present study, all phylogenetic trees were distance-based, however, the use of character-based phylogenies is becoming increasingly common among transmissions studies (Bowers et al., 2018; Manara et al., 2018; Planet et al., 2017). As both tree types make certain evolutionary assumptions, the validity of which remains unclear, it is perhaps advisable to include both a distance-based (e.g. NJT) and character-based (e.g. MLT) phylogenetic tree when designing a comprehensive analysis pipeline (Aanensen et al., 2016; Roisin et al., 2016).

In Chapter 6, wgMLST was used to identify clusters representing recent transmission, among a large group of isolates. This straightforward strategy exemplifies that which could be implemented in a clinical setting. Although wgMLST is not necessarily appropriate for distantly related isolates, clinical laboratories would perform comparative genomics solely to identify intra-hospital transmission events and thus, the relatedness of the remaining isolates would be of little importance. The routine employment of this relatively uncomplicated technique may therefore be practical in such settings. Nonetheless, research laboratories should continue to employ and investigate more sophisticated SNV-based analysis methods.

The issue of WGS data interpretation arose in all three parts of this study that investigated local transmission, i.e. Chapters 3, 5 and 6. A sub-study performed in Chapter 3 revealed that ST1-MRSA-IV recovered from a swab sample from a single individual could exhibit

up to 17 cgMLST allelic differences. Similarly, the Chapter 6 study participants yielded isolates belonging to a range of STs which exhibited up to 17 wgMLST allelic differences. This type of intra-host strain diversity is often viewed as a limitation on WGS-based transmission studies (Harris et al., 2013; Worby et al., 2014). Interestingly, however, a recent study demonstrated that intra-host strain variation could be exploited to determine whether a long-term carrier was involved in an outbreak (Gordon et al., 2017). Firstly, this study showed that intra-host strain diversity tends to develop over time, i.e. those who have recently acquired a strain will exhibit a small so-called cloud of diversity (Harris et al., 2013), while long term carriers will harbour a much more diverse population (Gordon et al., 2017). This highlights the limitations associated with the use of simple relatedness thresholds. Secondly, this study demonstrated that outbreaks which had evidence of a long-term carrier (e.g. colonised/infected patients had non-overlapping ward stays), yielded a much more diverse set of isolates than those without evidence of a long-term carrier (Gordon et al., 2017). In Chapters 3 and 5, the outbreak isolates exhibited up to 80 SNVs and 71 wgMLST allelic differences, respectively. These significant levels of diversity indicate the involvement of a long-term carrier(s) in each outbreak, most likely a HCW, as suggested by the findings in each chapter.

7.4 The future of WGS in microbiology

Whole-genome sequencing has the potential to replace virtually all current microbiological methods in both surveillance and clinical laboratories. At the outset of this study, most of the evidence supporting the use of WGS in clinical and public health microbiology derived from retrospective studies. Recently, however, some European reference laboratories have started to employ WGS on a routine basis. *Neisseria meningitidis* is currently the top bacterial surveillance target for national WGS-based surveillance, followed by CRE, and then MRSA (ECDC, 2016). The employment of WGS by these public health laboratories has facilitated the identification of various technical requirements that must be met for each pathogen in order to harmonise the widespread adoption of WGS-based typing in Europe (ECDC, 2016).

The first major pre-requisite for inter-laboratory comparability is the generation of quality assurance parameters. All sequencing data produced by public health laboratories must reach a minimum standard (ECDC, 2016). Secondly, a WGS analysis strategy must be agreed upon. The European Centre for Disease Control has indicated that this will likely involve two steps, one of which will enable genotypic nomenclature (e.g. cgMLST), and

the other of which will permit detailed phylogenetic reconstruction within genotypes (e.g. SNV analysis) (ECDC, 2016). Thirdly, empirical data interpretation criteria must be established (ECDC, 2016), which take into account all relevant research regarding the complexity of this issue. Fourthly, international data storage and exchange platforms must be developed (ECDC, 2016). Fifthly, a high-security system must be developed whereby epidemiological and clinical data can be linked (ECDC, 2016). Finally, significant investments must be made to facilitate access to WGS instruments, validated bioinformatic tools, laboratory infrastructure and operational funding (ECDC, 2016).

Efforts to date have been largely focussed on implementing routine WGS in surveillance laboratories. The next step will be to introduce this technology into hospital laboratories for real-time patient care. The workflow and priorities relating to the implementation of WGS in clinical laboratories differ slightly from those in reference facilities. Firstly, the species will need to be identified, necessitating an additional step at the start of the data analysis pipeline. Secondly, the isolates will be compared only to other isolates in the hospital, possibly permitting the use of a simplified (and faster) data analysis strategy. Thirdly, the turn-around-time (TAT) will be of greater importance as the data generated will be used to inform both treatment and IPC decisions. This will likely be facilitated by the use of an automated analysis pipeline. Finally, all relevant information should be clearly presented to the physician in a single report per patient. Although no currently published studies have trialled this entire process, a recent study demonstrated that NGS-based typing could be used to inform IPC decisions in a large German hospital, with a TAT of 4.4-5.3 days (Mellmann et al., 2016). While this study did not use WGS to predict resistance phenotypes, previous studies have demonstrated that this is a feasible alternative to conventional susceptibility testing (Gordon et al., 2014; Zankari et al., 2013).

As technologies improve, the TATs associated with WGS-based typing will decrease dramatically. For example, the use of a more advanced ONT system in a clinical setting would permit the preliminary identification of important resistance genes before the sequencing run is complete. Furthermore, the prospect of fourth generation sequencing is becoming more likely (Bachmann et al., 2018; Brown et al., 2015; Seth-smith et al., 2013). This involves genome sequencing directly from the clinical sample, negating the requirement for isolate culture and DNA extraction. The use of this technology would drastically decrease TATs and resolve the issues surrounding the processing of slow growing organisms such as *Mycobacterium tuberculosis*.

7.5 Concluding remarks

The widespread use and misuse of antibiotics has led to emergence and global dissemination of MRSA and MDR-MRSA. As antibiotic treatment options become increasingly limited, it is essential that the spread of these pathogens is effectively contained. The advent of WGS has provided the technology required to accurately track the spread of MRSA and other important pathogens. The challenge is now to improve upon these technologies and establish them as routine clinical microbiology tools so that they may be used to directly benefit both public health and patient care. While this will involve significant co-operation and investment at both national and international levels, failure to exploit these revolutionary advances will have serious and long-lasting consequences for human health worldwide.

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Appendix 1

PLOS ONE

RESEARCH ARTICLE

The recent emergence in hospitals of multidrug-resistant community-associated sequence type 1 and *spa* type t127 methicillin-resistant *Staphylococcus aureus* investigated by whole-genome sequencing: Implications for screening

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. The nucleotide sequence of the ST1-MRSA-IV isolate M14/0355 ileS2-encoding plasmid (p140355) has been submitted to GenBank (accession number KY465818).

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Abstract

Community-associated *spa* type t127/t922 methicillin-resistant *Staphylococcus aureus* (MRSA) prevalence increased from 1%-7% in Ireland between 2010–2015. This study tracked the spread of 89 such isolates from June 2013-June 2016. These included 78 healthcare-associated and 11 community associated-MRSA isolates from a prolonged hospital outbreak (H1) ($n = 46$), 16 other hospitals ($n = 28$), four other healthcare facilities ($n = 4$) and community-associated sources ($n = 11$). Isolates underwent antimicrobial susceptibility testing, DNA microarray profiling and whole-genome sequencing. Minimum spanning trees were generated following core-genome multilocus sequence typing and pairwise single nucleotide variation (SNV) analysis was performed. All isolates were sequence type 1 MRSA staphylococcal cassette chromosome *mec* type IV (ST1-MRSA-IV) and 76/89 were multidrug-resistant. Fifty isolates, including 40/46 from H1, were high-level mupirocin-resistant, carrying a conjugative 39 kb *iles2*-encoding plasmid. Two closely related ST1-MRSA-IV strains (I and II) and multiple sporadic strains were identified. Strain I isolates (57/89), including 43/46 H1 and all high-level mupirocin-resistant isolates, exhibited $\leq$80 SNVs. Two strain I isolates from separate H1 healthcare workers differed from other H1/strain I isolates by 7–47 and 12–53 SNVs, respectively, indicating healthcare worker involvement in this outbreak. Strain II isolates (19/89), including the remaining H1 isolates, exhibited $\leq$127 SNVs. For each strain, the pairwise SNVs exhibited by healthcare-associated and community-associated isolates indicated recent transmission of ST1-MRSA-IV within and between multiple hospitals, healthcare facilities and communities in Ireland. Given the interchange between healthcare-associated

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and community-associated isolates in hospitals, the risk factors that inform screening for MRSA require revision.

Introduction

Staphylococcus aureus can cause a wide variety of diseases ranging in severity from superficial skin infections to life-threatening invasive infections such as necrotizing pneumonia, endocarditis and sepsis [1, 2]. Methicillin-susceptible *S. aureus* become methicillin-resistant *S. aureus* (MRSA) upon acquisition of the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) mobile genetic element. SCC*mec* harbors *mecA* or *mecC*, both of which encode alternate penicillin-binding proteins, which mediate resistance to almost all β-lactam antibiotics [3–5]. The *mecA* gene encodes the penicillin-binding protein known as PBP2a, whereas *mecC* encodes a homolog that shares 62% amino acid identity with MecA proteins previously described in MRSA [6, 7]. MRSA constitute a major burden in healthcare and community settings worldwide.

Accurate characterization and tracking of nosocomial MRSA strains is essential to reduce the spread of infection. Previously, sequence-based typing approaches for MRSA focused on molecular typing methods that characterize small sections of the genome including multilocus sequence typing (MLST), SCC*mec* and *spa* typing [8, 9]. However, in recent years whole-genome sequencing (WGS) has revolutionized tracking the spread of MRSA in both outbreak and long-term epidemiological investigations [10, 11]. Analysis of single nucleotide variations (SNVs) between isolates provides higher-level discrimination compared to traditional molecular typing techniques, although data analysis involves complex bioinformatics [10, 12–14]. Data from *S. aureus* sequence types (STs) ST22, ST2257, ST30 and ST36 showed that multiple colonies recovered from a single patient swab can vary by up $\leq$40 SNVs [15]. This 40 SNV intra-host strain variation threshold has since been used to infer relatedness between isolates and to identify transmission events [10, 16]. In addition to SNV analysis, whole-genome MLST (wgMLST), involving >1,800 genome-wide loci, has been applied to investigate relationships between MRSA isolates [17]. This approach currently provides the optimal resolution to infer phylogenetic relatedness among isolates, permitting the identification of possible, probable, or unlikely cases of epidemiological linkage. Core-genome MLST (cgMLST), which excludes accessory genome loci, is a refinement of wgMLST based on genes present in each isolate genome [18].

MRSA are largely categorized as healthcare-associated (HCA) and community-associated (CA). While HCA-MRSA often exhibit resistance to multiple antimicrobial agents and typically infect individuals who are immunocompromised or have specific risk factors, CA-MRSA have traditionally been associated with colonization/infection of healthy individuals and susceptibility to most antibiotics [19]. In recent years however, distinctions between these groups have become blurred. CA-MRSA clones have become prevalent in some nosocomial settings [20, 21] and multidrug-resistant (MDR) CA-MRSA are being increasingly reported [22, 23]. Furthermore, genetic markers including SCC*mec* IV and V or virulence determinants such as the Panton-Valentine leukocidin (PVL) toxin, previously considered to be exclusively associated with CA-MRSA, are no longer reliable indicators [22, 24–26].

Since its emergence in the 1990s as the first CA-MRSA clone [27], ST1-MRSA-IV has arisen in diverse settings. Following its initial success as a CA clone [19, 28], ST1-MRSA-IV has been associated with HCA-colonization and infection in North and South America, Europe, the Middle East and Asia [21, 29–31]. More recently, ST1-MRSA-IV *spa* type (t) 127 has been recovered from companion animals, livestock and livestock produce in Italy, Austria and Hungary [32–36].

MRSA has been endemic for four decades in Irish hospitals, since first reported in 1971 [10, 24, 37–43]. While predominant MRSA clone replacement has occurred several times in Ireland [40], ST22-MRSA-IV has been the predominant nosocomial clone since 2002 [10, 44]. Characterization of sporadically-occurring MRSA in Ireland between 2000 and 2012 identified an extensive range of MRSA genotypes and the emergence of several PVL-negative CA-MRSA clones, including ST1-MRSA-IV-t127, which accounted for just 2.3% (2/88) of isolates [40]. In 2010, <1% of all isolates identified at the Irish National MRSA Reference Laboratory (NMRSARL) were t127, or the closely related t922, while in 2015, these isolates accounted for 7% of all those detected [45, 46].

This study comprehensively characterized 89 MRSA-t127/t922, isolates, recovered between 2013–2016 from multiple hospital, healthcare and community sources in Ireland, including a protracted hospital outbreak, in order to investigate isolate relationships and the extent of their spread. Core-genome MLST and SNV analyses revealed the recent emergence and extensive spread of two closely related strains and multiple sporadic strains of ST1-MRSA-IV-t127/t922. Isolates of this clone were predominantly MDR and frequently high-level mupirocin resistant (Hi-MupR), the latter of which can negatively affect efforts to eradicate carriage in colonized individuals.

Results

MRSA isolates

Eighty-seven t127 and two t922-MRSA isolates identified by the NMRSARL from June 2013-June 2016 were investigated. The majority of isolates (78/89; 87.6%) were HCA-MRSA, 46/78 (59.0%) of which were recovered from infections or colonization screening during a protracted outbreak in a single hospital (H1) from November 2013-February 2016. The remaining HCA-MRSA isolates (32/78; 41.0%) were recovered in 16 separate Irish hospitals (H2-H17) and four other healthcare facilities (HCFs). Eleven isolates (11/89, 12.4%) were CA-MRSA. Four isolates from hospitals/HCFs other than H1 and one CA-MRSA isolate were recovered from patients with recent hospital H1 admission history (see S1 Table for details). The majority of isolates (75/89; 84.3%) were MDR, exhibiting phenotypic resistance to three or more clinically relevant antibiotic classes in addition to β-lactams, including aminoglycosides, macrolides, mupirocin, tetracycline and fusidic acid (Table 1). DNA microarray profiling confirmed the presence of corresponding resistance genes including *aphA3* (79/89 isolates; 88.8%)

Table 1. Phenotypic resistance patterns of 89 ST1-MRSA-IV t127/t922 MRSA isolates investigated to six clinically relevant antibiotic classes.

Isolate source (no. of isolates)	No. of isolates exhibiting resistance to antibiotic classes (%)[a]						
	BL	AG	ML	MUP	TET	FUS	MDR[b]
Healthcare-associated (78)	78 (100)	70 (89.7)	72 (92.3)	46 (59.0)	67 (85.9)	10 (12.8)	66 (84.6)
Hospital 1 (46)	46 (100)	41 (89.1)	41 (89.1)	40 (87.0)	38 (82.6)	1 (2.2)	38 (82.6)
Hospitals 2–17 and four other HCFs (32)	32 (100)	29 (90.6)	31 (96.9)	6 (18.8)	29 (90.6)	9 (28.1)	28 (87.5)
Community-associated (11)	11 (100)	9 (81.8)	9 (81.8)	4 (36.4)	8 (72.7)	1 (9.1)	9 (81.8)
Total (89)	89 (100)	79 (88.8)	81 (91.0)	50 (56.2)	75 (84.3)	11 (12.4)	75 (84.3)

[a]In each row, all percentages are expressed as a proportion of the total number of isolates investigated.

[b]Multidrug-resistant (MDR) isolates were defined as those exhibiting phenotypic resistance to three or more classes of clinically relevant antibiotics in addition to β-lactams.

Abbreviations: AG, aminoglycoside antibiotics; BL, β-lactams; FUS, fusidic acid; HCFs, healthcare facilities; ML, macrolide/lincosamides; MUP, mupirocin; TET, tetracycline.

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and *aadD* (1/89; 1.1%) encoding aminoglycoside resistance, *erm*(C) (71/89; 79.8%) encoding macrolide resistance, *mupA (ileS2)* (52/89; 58.4%) encoding high-level mupirocin resistance, *tet*(K) (39/89; 43.8%) and *tet*(M) (1/89; 1.1%) encoding tetracycline resistance and *fusB* (2/89; 2.2%) and *fusC* (7/89; 7.9%) encoding fusidic acid resistance. The majority of isolates (55/89; 61.8%) harbored at least one *qac* gene encoding resistance to quaternary ammonium compounds. DNA microarray profiling also confirmed that all isolates belonged to clonal complex (CC) 1, harbored SCC*mec* IV (CC1-MRSA-IV) and carried the enterotoxin gene *seh*, which is typically associated with CC1-MRSA. The immune evasion cluster (IEC) genes *sak* and *scn* (IEC type E) were detected in 88/89 (98.9%) isolates. Isolate details are shown in S1 Table.

CC1-MRSA-IV isolate relatedness based on WGS

All 89 isolates were assigned to ST1. Based on cgMLST, the majority of isolates (78/89; 87.6%) grouped within one major minimum spanning tree (MST) cluster, while outlier isolates (11/89; 12.4%) dispersed throughout the remainder of the MST (Fig 1). The 78 major MST cluster isolates, both HCA (68/78; 87.2%) and CA (10/78; 12.8%), were recovered over three years and differed from each other by 0–127 SNVs. *In vivo* SNV analysis revealed that two sets of 13 colonies, each isolated from a single patient swab (the patients from which study isolates M13/0653 and M15/0221 were recovered, respectively), differed by 0–36 SNVs and 0–43 SNVs, respectively. Based on this maximum intra-strain difference of 43 SNVs, isolates within the major MST cluster, recovered over three-years and differing by $\leq$127 SNVs, were deemed closely related. The majority of MDR isolates were located in the major MST cluster (74/75, 98.6%).

Two sub-clusters and two "intra-cluster outliers" were identified within the major MST cluster following the generation of a second MST based on cgMLST loci from isolates within the major MST cluster only (Fig 2). Isolates within each sub-cluster differed from each other at $\leq$58 cgMLST loci. Isolates within sub-clusters I and II differed by 0–80 and 2–127 SNVs, respectively and included all 46 H1 outbreak isolates (Fig 2). All isolates in sub-cluster I (57/57) and 89.5% (17/19) of those in sub-cluster II were MDR.

Sub-cluster I (Fig 2) consisted of 57 isolates recovered over 28-months (November 2013–March 2016), including the majority of H1 outbreak isolates (43/46; 93.5%) and isolates recovered from hospitals H3 (n = 4), H4 (n = 2), H13 (n = 1), H17 (n = 1), other HCFs (n = 2) and community sources (n = 4). Sub-cluster I isolates differed from each other by an average of 26 SNVs (range: 0–80 SNVs) (Fig 2). Isolates from H1 differed from each other by an average of 23 SNVs (range: 0–70 SNVs). Isolate M14/0992, recovered from a HCW in H1 and isolate M15/0213, recovered from another HCW in H1 who presented to a community-based general practitioner (GP), were both included in sub-cluster I. They differed from all other H1/sub-cluster I isolates by 12–53 SNVs and 7–47 SNVs, respectively. Sub-cluster I also included two isolate pairs, recovered from separate patients at different times, which exhibited intra-pair differences in susceptibility to mupirocin. Isolates M15/0148 (mupirocin-susceptible) and M15/0637 (Hi-MupR) from patient one were recovered nine months apart and differed by 33 SNVs, while isolates M15/0540 (mupirocin-susceptible) and M15/0541(Hi-MupR) from patient two were recovered 23 days apart and differed by four SNVs (Fig 2). The only environmental isolate investigated, which was recovered from hospital H1, was located in sub-cluster I. This differed from all other H1 isolates by 14–59 SNVs. The four CA isolates within sub-cluster I differed from H1/sub-cluster I isolates by 3–80 SNVs and from isolates from other hospitals/HCFs in sub-cluster I by 5–67 SNVs.

Sub-cluster II consisted of 19 isolates recovered over 36 months (June 2013–June 2016), including the remaining H1 isolates (3/46; 6.5%) and isolates from hospitals H2 (n = 2), H3 (n = 1), H4 (n = 2), H5 (n = 2), H8 (n = 1), H11 (n = 1), H 15 (n = 1), a nursing home (n = 1)

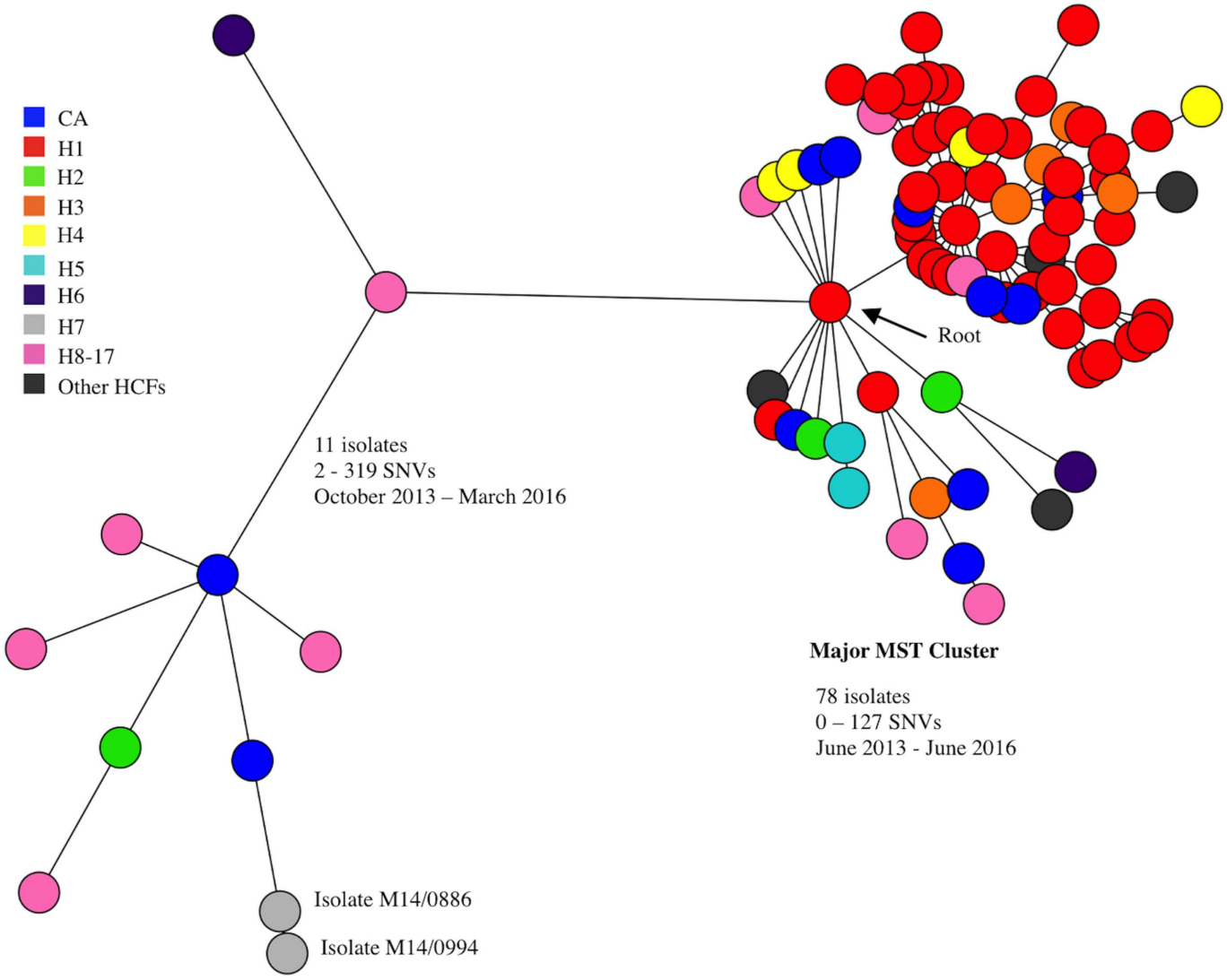

Fig 1. A minimum spanning tree based on core-genome multilocus sequence typing data from 89 ST1-MRSA-IV/t127 or t922 isolates. The pairwise single nucleotide variation (SNV) range between isolates inside and outside of the major minimum spanning tree (MST) cluster and their recovery time frame are indicated. Two outlier isolates, M14/0994 and M14/0886, were recovered 49 days apart from different patients on separate wards in hospital H7 and differed from each other by only two SNVs. The locations from which the isolates were recovered are indicated in the color legend. One isolate was recovered from each of hospitals H8-H17. Abbreviations: CA, community associated; H, hospital; HCFs, other healthcare facilities.

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and community sources ($n = 5$) (Fig 2). Isolates M14/0845 and M14/0857, recovered on the same day from different patients on the same ward of hospital H5, differed by two SNVs. The CA isolate M16/0002 and hospital H6 isolate M16/0116 were recovered 43 days apart and differed by six SNVs. Apart from these two instances, isolates within sub-cluster II differed from each other by 42–127 SNVs. The H1 isolates in sub-cluster II differed by 42–121 SNVs. The intra-cluster outlier isolates, M14/0597 and M16/0223, branched off from sub-cluster II (Fig 2). Both isolates were HCA and differed from sub-cluster II isolates by 91–149 and 79–128 SNVs, respectively.

The 11 isolates outside of the major MST cluster (outliers) were recovered over 29 months (October 2013–March 2016) and included one isolate from each of hospitals H2, H6, H9, H10,

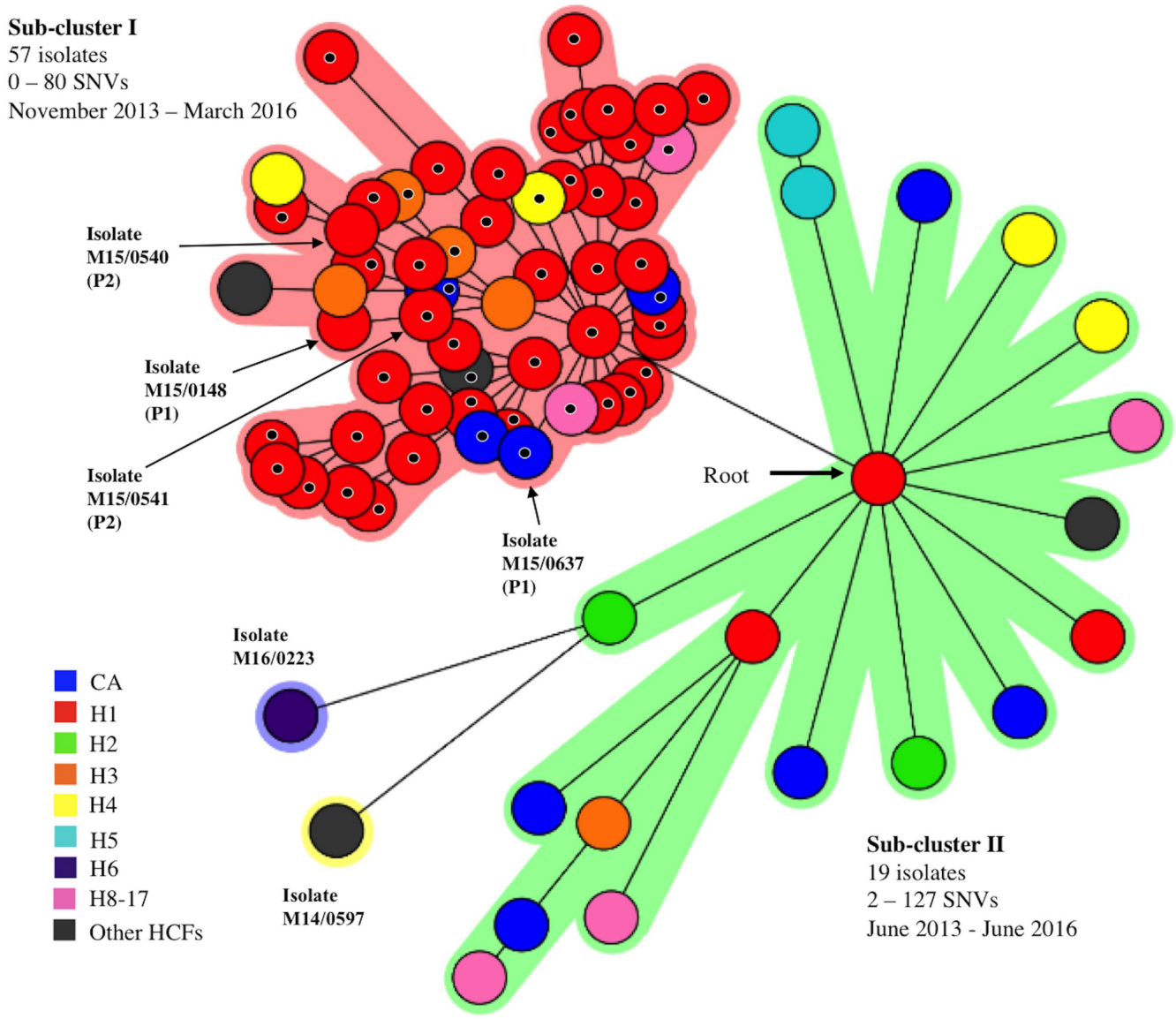

Fig 2. A minimum spanning tree based on core-genome multilocus sequence typing data from 78 ST1-MRSA-IV/t127 or t922 isolates within the major cluster identified in Fig 1. Two sub-clusters were evident within the major cluster: sub-cluster I; highlighted in pink and sub-cluster II; highlighted in green. The pairwise single nucleotide variation (SNV) range between isolates within each sub-cluster and their recovery time frame are indicated. Two intra-cluster outliers were present (M16/0223 and M14/0597), highlighted in purple and yellow, respectively. Two isolate pairs, each recovered from separate patients (patient P1, M15/0148; *iles2*-negative/mupirocin-susceptible and M15/0637; *iles2*-positive/high-level mupirocin-resistant) and patient P2 (M15/0540; *iles2*-positive/mupirocin-susceptible and M15/0541; *iles2*-positive/high-level mupirocin-resistant) are indicated using arrows. M15/0540 harbored an *iles2*-encoding plasmid with a premature stop codon within the *iles2* gene. The locations from which the isolates were recovered are indicated in the color legend. One isolate was recovered from each of hospitals H8-H17. Isolates that exhibited high-level mupirocin resistance are indicated using a small black circle overlaying the relevant isolate node. Abbreviations: CA, community associated; H, hospital; HCFs, other healthcare facilities.

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H12, H14 and H16, and two isolates from both hospital H7 and community sources (Fig 1). Two outlier isolates, M14/0994 and M14/0886 (Fig 1), were recovered 49 days apart from different patients on separate wards in hospital H7 and differed by two SNVs. Excluding this pair of isolates, the outliers differed from each other by 50–319 SNVs.

High-level mupirocin resistance

Phenotypic high-level mupirocin-resistance was detected in 50/89 (56.2%) isolates, all of which were *ileS2*-positive and located in MST sub-cluster I (Fig 2). A total of 46/50 (92.0%) Hi-MupR isolates were HCA and 4/50 (8%) were CA. The majority of HCA Hi-MupR isolates (40/46; 87.0%) were from H1 and accounted for 87.0% (40/46) of all H1 outbreak isolates. The remaining 6/46 (8.7%) HCA Hi-MupR isolates were from hospitals H3 (n = 2), H4 (n = 1), H13 (n = 1), H17 (n = 1) and a long-term care facility (n = 1). DNA microarray profiling detected the *ileS2* gene in two phenotypically mupirocin-susceptible isolates (M15/0201 and M15/0540) within MST sub-cluster I. A single adenine insertion at nucleotide position 283 in *ileS2* was identified in each isolate, resulting in a downstream frameshift mutation and a premature stop codon. Twenty-three days after the recovery of isolate M15/0540, a phenotypically Hi-MupR isolate (M15/0541) harboring *ileS2* without the adenine insertion was recovered from the same patient (P1, Fig 2).

A single *ileS2*-encoding plasmid in all Hi-MupR isolates

Mating and plasmid curing experiments using the Hi-MupR isolate M14/0355 confirmed the presence of a conjugative *ileS2*-encoding plasmid. DNA microarray profiling confirmed the gain of *ileS2* in transconjugants and its loss in cured derivatives, which were Hi-MupR (mupirocin MIC >1024 mg/L) and mupirocin-susceptible (mupirocin MIC <1 mg/L), respectively. A BLAST analysis of the single-molecule real-time (SMRT) derived sequence of the *ileS2*-encoding plasmid of M14/0355 revealed that it shared 99% DNA sequence identity with *iles2*-encoding plasmid, pV030-8 (GenBank accession number: NC_010279). Successful alignment of both the sequence reads and contigs of the remaining 49 Hi-MupR isolates to the SMRT sequence of the *ileS2*-encoding plasmid of M14/0355 confirmed the presence of the pV030-8-like plasmid in all 50 Hi-MupR isolates. The SMRT sequence of the M14/0355 *ileS2*-encoding plasmid (p140355) has been submitted to GenBank (accession number: KY465818).

Carriage of SCC*fus*

DNA microarray profiling showed that SCC*fus*, encoding fusidic acid resistance, was only carried by the outlier isolates (7/11; 63.7%), both HCA (n = 6) and CA (n = 1) (Fig 1).

Discussion

This study revealed the recent emergence and extensive spread of several strains of a predominantly MDR CA-MRSA clone, CC1-ST1-MRSA-IV, within and between hospitals/HCFs and communities throughout Ireland. Its resistance to many clinically relevant antibiotics (Table 1), which restricts patient treatment options and its often Hi-MupR nature, which eliminates the option of mupirocin nasal decolonization, merit particular concern. Interestingly, increased prevalence of a MDR CA-MRSA clone, *pvl*-positive CC1-ST772-MRSA-V, was previously reported in Ireland [22]. Although this clone did not spread as extensively as the CC1-ST1-MRSA-IV clone investigated here, this pattern reflects the relatively recent worldwide trend of CA-MRSA spreading into hospitals. Worryingly, the MDR CA-MRSA clone investigated here was detected in 17 hospitals, four other HCFs and from 11 people in the community, over the last three years (2013–2016). The extensive spread of this CA-MRSA clone within and between the Irish community and hospitals/HCFs highlights the need for infection prevention and control measures that consider CA-MRSA transmission routes into hospitals. While current Irish National Clinical Guidelines for infection prevention and control of MRSA recommend screening at-risk patients, only HCA-MRSA risk factors are

considered [47]. Additionally, routine screening of HCWs, who are a potential source of CA-MRSA, is not mandatory in Ireland, except during outbreaks.

The use of cgMLST analysis grouped the majority of isolates (78/89) into one major MST cluster, leaving just 11 outliers. Pairwise SNV comparison subsequently provided enhanced discrimination between isolates. In order to inform our SNV comparison interpretation, we considered the SNV analysis data sets associated with multiple ST1-MRSA-IV isolates derived from single patient swabs, which indicated that a difference of $\leq$43 SNVs could be deemed negligible when assessing relatedness between ST1-MRSA-IV isolates. This maximum intra-host strain variation estimation conforms with that of 40 SNVs, previously established for *S. aureus* belonging to ST22, ST2257, ST30 and ST36 [15]. In some previous studies, this estimation has been used as a relatedness-threshold, with isolates differing by $\leq$40 SNVs being deemed closely related [10, 16]. However, given the external pressures to which isolates from the present study were presumably subjected during the three-year period in which they were recovered, this 40 SNV relatedness threshold was deemed inappropriate. Considering this, combined with the assumption that the MST indicated the most probable relationship between isolates, or at least the presence of isolate groups, the major MST cluster isolates (Fig 1), which differed by a maximum of 127 SNVs, were deemed closely related. Thus, the contemporaneous circulation of two closely related strains, and multiple sporadic strains, was identified (Fig 2).

Strain I isolates (sub-cluster I, Fig 2) were identified mainly in hospital H1 (43/46 isolates) but also in four other hospitals, two HCFs and from the community. Upon further investigation of strain I, it was found that H1 isolates differed from isolates from other hospitals/HCFs by as few as three SNVs and that the CA isolates differed from H1 isolates and isolates from other hospitals/HCFs by as few as three and five SNVs, respectively. Although definitive conclusions cannot be drawn regarding the original source(s) of this strain, these data clearly indicate that strain I spread within and between five different hospitals, two HCFs and the community. Interestingly, two H1 HCWs carried strain I isolates differing from other H1 isolates by as few as seven and 12 SNVs, respectively. It therefore cannot be ruled out that HCWs acted as a reservoir for this strain during the outbreak. Travel of staff between healthcare facilities is common in Ireland and this, combined with the transfer of patients between hospitals/HCFs, likely contributed to the dissemination of strain I. The use of mupirocin in hospital H1 may have driven selection of Hi-MupR strain I isolates. Hospitals in Ireland follow national guidelines for patients and healthcare staff found to be colonized with MRSA [47]. Attempts at decolonization may be considered for colonized patients who are due to undergo an elective operative procedure, patients in a clinical area where there is a high risk of colonization leading to invasive infection, if the risk of infection is high and the consequences severe (e.g. immunocompromised patients), or as part of a strategy to address uncontrolled transmission despite the use of other measures. National guidelines for MRSA decolonization recommend the use of nasal treatment with mupirocin and a chlorhexidine body wash. Interestingly, 53/57 (93%) strain I isolates also harbored *qac* genes encoding resistance to quaternary ammonium compounds such as chlorhexidine (S1 Table).

Strain II isolates (sub-cluster II, Fig 2) were recovered from eight different hospitals, one nursing home and the community. This strain, although represented by fewer isolates (strain I, $n = 57$; strain II, $n = 19$), was more divergent than strain I, exhibiting a higher SNV range (strain I, 0–80 SNVs; strain II, 0–127 SNVs). A difference of just six SNVs between H6 isolate, M16/0116, and CA isolate, M16/0002, indicated that strain II transmission between a nosocomial and community setting had occurred in at least one instance.

Eleven ST1-MRSA-IV-t127 isolates, not assigned to strain I or II (outlier isolates, Fig 1) were recovered from eight hospitals and the community in Ireland during the time period in which strains I and II circulated. It is possible that the predominantly MDR nature of strains I

and II, lacking in the majority (10/11) of outlier isolates, may have facilitated their spread. Although rarely MDR, 63.6% of outlier isolates harbored SCC*fus* and exhibited fusidic acid resistance, suggesting that fusidic acid usage may have encouraged selection of these strains. While systemic use of fusidic acid has decreased in Ireland in recent years [48], topical use of fusidic acid in the community may have contributed towards selection of these strains.

The *ileS2*-encoding plasmid, p140355, carried by all Hi-MupR MRSA isolates exhibited 99% DNA sequence identity to the previously described pV030-8 plasmid, (GenBank accession number: NC_010279, direct submission) identified in 2007 in South Korea. Reports of this plasmid in the literature however, are lacking and its global prevelance is unknown. At 39 kb, p140355 is approximately 2.7 kb smaller than the more commonly reported *ileS2*-encoding pPR9 plasmid [49].

Several studies reported the displacement of previously predominant HCA- by CA-MRSA clones, highlighting the importance of continued monitoring and surveillance of CA-MRSA both in hospitals and communities. In India, the MDR CA and *pvl*-positive CC1-ST772-MRSA-V clone displaced the previously predominant HCA ST239-MRSA-III clone [8, 50]. In the USA, the CA ST8-MRSA-IV clone USA300 now constitutes the leading cause of MRSA nosocomial infections, having overtaken the previously predominant HCA ST5-MRSA-II clone, USA100 [51]. While ST22-MRSA-IV continues to predominate as the major cause of nosocomial MRSA infections in Ireland, ST1-MRSA-IV isolates represented the second most common clone identified by the NMRSARL in 2015 [46]. Hospital outbreaks involving ST1-MRSA have been reported elsewhere in Europe including the UK, Denmark and Italy in 2006, 2008 and 2012, respectively [52–54]. The emergence of ST1 MRSA in nosocomial settings is not exclusively confined to outbreak scenarios; in 2015 ST1-MRSA was the most common clone circulating in seven nursing homes in Shanghai, China, accounting for 29.1% of MRSA [31].

The emergence of a predominantly MDR CA-MRSA clone and its subsequent dissemination into hospitals, HCFs and the community throughout Ireland is worrying. Infection prevention and control measures should consider CA-MRSA risk factors and not only HCA-MRSA risk factors during MRSA screening and should recognize the importance of screening HCWs for MRSA.

Materials and methods

Ethics statement

None of the work described in this manuscript involved human subjects or work on animals. The study investigated MRSA isolates from patients submitted to NMRSARL. No patient identifying information whatsoever is contained in the manuscript.

Isolates

In 2010, 0.18% of all MRSA isolates identified at the NMRSARL were mupirocin-susceptible MRSA-t127. However, in 2015 4.49% and 2.59% of all isolates identified at the NMRSARL were mupirocin-susceptible and Hi-MupR MRSA-t127, respectively. Eighty-seven *spa* type t127-MRSA and two *spa* type t922-MRSA isolates identified at the NMRSARL between June 2013 and June 2016 were investigated in the present study (S1 Table). All isolates were *pvl*-negative. Isolates were deemed to be HCA if they were recovered from hospital in-patients at least 48 h post-admission ($n = 72$), the hospital environment ($n = 1$), from hospital healthcare workers ($n = 2$) or from residents in nursing homes ($n = 2$) and long-term care facilities ($n = 1$). Isolates were deemed to be CA-MRSA if they were recovered from patients attending community-based GPs ($n = 9$) and hospital accident and emergency ($n = 1$) and outpatient departments ($n = 1$). Forty-six of the HCA

isolates were recovered from colonized or infected sites of in-patients ($n = 44$), a colonized health-care worker ($n = 1$) or from the environment ($n = 1$) in an 820-bed acute care hospital in Dublin, Ireland (H1) during a protracted t127-MRSA outbreak between November 2013 and February 2016 involving patients from 11 wards (29 isolates were from one ward (ward A)). Outbreak isolates were initially detected from clinical samples. Further cases were identified from screening specimens taken from patients with risk factors for MRSA colonization (such as previous history of MRSA colonization/infection at hospital H1) and from clinical samples. Following the identification of the outbreak, H1 in-patients in the affected areas were subjected to active screening for MRSA. Thirty-two of the HCA isolates were recovered between October 2013 and March 2016 from in-patients in 16 other hospitals (H2-H17) or from other HCFs. Only one isolate per patient was included in this study with the exception of three pairs of isolates from separate patients: patient one isolates, M15/0148 (H1, ward E; mupirocin-susceptible and *ileS2*-negative) and M15/0637 (recovered by patient's GP; Hi-MupR and *ileS2*-positive); patient two isolates, M15/0540 (H1, ward A; phenotypically mupirocin-susceptible but *ileS2*-positive) and M15/0541 (H1, ward I; Hi-MupR and *ileS2*-positive), patient three isolates, M14/0965 (H1, ward A; Hi-MupR and *ileS2*-positive) and M15/0223 (H1, ward B; Hi-MupR and *ileS2*-positive). Isolates from patients one to three were recovered nine months, 23 days and four months apart, respectively (S1 Table). Furthermore, multiple colonies recovered from two separate patient swabs (same patients that yielded M13/0653 and M15/0221) were used to determine intra-strain variation *in vivo* (see below).

Isolates were identified as *S. aureus* using the tube coagulase test and methicillin resistance was detected using 30-μg cefoxitin disks (Oxoid Ltd., Basingstoke, United Kingdom) in accordance with European Society of Clinical Microbiology and Infectious Diseases (EUCAST) methodology and interpretive criteria [55]. MRSA isolates were stored at -80˚C on individual Protect Bacterial Preservation System cryogenic beads (Technical Services Consultants Ltd., Heywood, United Kingdom).

Antimicrobial susceptibility testing

The susceptibility of all isolates was determined against a panel of 23 antimicrobial agents and heavy metals by disk diffusion using EUCAST methodology and interpretative criteria [55]. If not available, Clinical Laboratory Standards Institute disk concentrations and interpretive criteria were used [56], or for the remaining agents (including all heavy metals tested), the disk concentrations and interpretive criteria of Rossney *et al*. were used [39]. The 23 agents tested were amikacin, ampicillin, cadmium acetate, chloramphenicol, clindamycin, ciprofloxacin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mercuric chloride, mupirocin, neomycin, phenyl mercuric acetate, rifampicin, spectinomycin, streptomycin, sulphonamide, tetracycline, tobramycin, trimethoprim and vancomycin.

The mupirocin MIC of each isolate was determined using mupirocin E-test strips (bioMérieux, Nuertlingen, Germany) according to the manufacturer's instructions. Following incubation for 24 h at 37˚C, the mupirocin MIC of each isolate was determined to be the nearest two-fold dilution, above which there was no visible growth. Isolates were deemed to be mupirocin susceptible if they exhibited a mupirocin MIC of $\leq$1 mg/L, to exhibit low-level mupirocin resistance if they had a mupirocin MIC of 2–128 mg/L, or to exhibit high-level mupirocin resistance if they exhibited a mupirocin MIC $\geq$ 256 mg/L [55].

Molecular typing of isolates

All isolates underwent *spa* typing and DNA microarray profiling. For *spa* typing, genomic DNA was extracted from isolates using a 6% InstaGene matrix solution according to the manufacturer's instruction (BioRad, München, Germany). Sequences were analyzed using the

Ridom StaphType software package version 1.5 (Ridom Gmbh, Wurzburg, Germany) and *spa* types were assigned using the SpaServer website (http://spaserver2.ridom.de).

Genomic DNA for DNA microarray profiling was extracted from each isolate by enzymatic lysis using the buffers and solutions provided with the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) and the DNeasy blood and tissue kit (Qiagen, Crawley, West Sussex, United Kingdom) according to the manufacturer's instructions. DNA microarray profiling was performed using the *S. aureus* Genotyping Kit 2.0 (Alere), which consists of individual DNA microarrays mounted in 8-well microtiter strips that detect 333 *S. aureus* gene sequences and alleles, including species-specific, antimicrobial resistance and virulence-associated genes, SCC*mec* genes and typing markers. ArrayMate software (version 2012-01-18) (Alere) was used to analyze data generated by the microarray system and to assign isolates to STs and/or CCs by comparing the microarray profile results of test isolates to the corresponding profiles of an extensive range of reference strains stored in the ArrayMate database that had previously undergone MLST [57]. The primers, probes, and protocols for the DNA microarray system have been described in detail previously [58].

Plasmid conjugation and curing

The plasmid-free novobiocin-resistant *S. aureus* laboratory strain XU21 was used as a plasmid recipient strain during filter mating experiments [59]. Conjugative transfer of the plasmid-encoded *ileS2* gene from the t127-MRSA isolate M14/0355 to the plasmid-free *S. aureus* recipient strain XU21 was performed by filter mating as described previously [59]. Presumptive transconjugant derivatives were selected by subculture on brain heart infusion (BHI) agar (Oxoid Ltd.) supplemented with mupirocin (100 μg/ml) (GlaxoSmithKline, Citywest Business Campus, Dublin, Ireland) and novobiocin (10 μg/ml) (Sigma-Aldrich) and were confirmed by DNA microarray profiling and mupirocin MIC determination.

Curing of the *ileS2*-encoded plasmid from isolate M14/0355 was performed following reactivation from a cryogenic bead on to a Tryptic Soy Agar plate and culturing one colony in 5 ml of Brain Heart Infusion (BHI) broth (Oxoid Ltd.) at 43˚C and 200 rpm for 24 h. This was followed by subculturing 0.1 ml into 5 ml of fresh BHI broth and incubation as before (43˚C and 200 rpm for 24 h) for four consecutive rounds. Individual colonies obtained following plating on BHI agar were screened for the loss of mupirocin resistance by replica plating onto BHI agar supplemented with mupirocin at 100 μg/ml and putative cured derivatives were confirmed by DNA microarray analysis.

Whole-genome sequencing

Genomic DNA for whole-genome sequencing was extracted using the Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions. Whole-genome sequencing was undertaken using the Nextera XT library preparation reagents in accordance with the manufacturer's instruction (Illumina, Eindhoven, The Netherlands). Libraries were sequenced on an Illumina MiSeq instrument. Resulting fastQ files were imported directly from Illumina BaseSpace to the BioNumerics (version 7.6) (Applied Maths, Belgium) cloud-based calculation engine, where they were assembled using the Velvet *de novo* genome assembler (version 1.2.10). Both the fastQ files and assembled genome of each isolate were submitted to the BioNumerics wgMLST scheme for assembly-free and assembly-based allele calling, respectively. To investigate relationships between isolates, a MST was generated using BioNumerics, based on core-genome loci, as previously described by Leopold *et al.* [18]. The genome of the centrally located isolate in the MST, M15/0029 (designated as the "root") (Fig 1), was chosen as the reference sequence against which all other isolate genomes were mapped. The

BioNumerics genome analysis tool was used to record SNVs between each isolate and the root, yielding a SNV matrix detailing all SNV positions in the pan genome. Using Clustal Omega, a multiple sequence alignment of the SNV matirix was carried out and an n × n percentange identity matrix was generated [60]. In order to calculate SNVs between all possible isolates pairs, a pairwise SNV matrix was created (S1 Dataset) by applying the following equation to the percentage identity matrix:

$$x = \frac{(100 - y)(n)}{100}$$

Where, x = the number of SNVs by which two isolates differ

y = the percentage identity of the SNV matrix sequence of two isolates

n = the total number of SNV positions in the pan genome

The ST of each isolate was also assigned using WGS data and the Ridom SeqSphere+ software package version 3.3.0 (Ridom GmbH, Germany).

Plasmid sequence analysis using WGS data

The Hi-MupR ST1-MRSA-IV-t127 isolate that underwent conjugation and curing (M14/0355) also underwent SMRT sequencing (Pacific Biosciences, Norwich, United Kingdom) in order to obtain the nucleotide sequence of the entire *ileS2*-containing plasmid on one contiguous sequence. The MiSeq-generated reads of the remaining Hi-MupR MRSA isolates were mapped to the M14/0355 SMRT sequence using the Burrows-Wheeler aligner (BWA-mem) (http://arxiv.org/abs/1303.3997). Artemis sequence viewer (http://www.sanger.ac.uk/science/tools/artemis) was used to visually assess the mapping of reads to the M14/0355 sequence. Contigs were generated by *de novo* assembly using the SPAdes assembler [61], were aligned to the M14/0355 SMRT sequence using the BWA-mem and were visualized using Artemis. Blast was used to search the literature for *ileS2*-encoding plasmids similar to that harbored by M14/0355 (https://www.blast.ncbi.nlm.nih.gov).

In vivo SNV investigation

In order to inform the interpretation of the WGS SNV data, the SNVs of two sets of 13 individual colonies cultured from separate patient swabs (the patients from which study isolates M13/0653 and M15/0221 were recovered, respectively) were investigated. Colonies were recovered from both swabs by plating on SaSelect chromogenic agar plates (BioRad) for isolation and initial identification of *S. aureus* following incubation at 37˚C for 24 h. Following incubation, 13 well separated individual colonies were each subcultured onto separate SaSelect chromogenic agar plates to obtain pure cultures. In each case, following confirmation of *S. aureus* identification by latex agglutination using the Pastorex™ Staph-Plus kit (Bio-Rad, Hercules, California, USA), one colony from each of the 13 plates was selected for *spa* typing and DNA microarray profiling. Genomic DNA was extracted using the Qiagen DNeasy blood and tissue kit and underwent WGS preparation and MiSeq sequencing, as described above. The BioNumerics genome analysis tool was used to record SNVs between the genomes of each colony, yielding a pan genome SNV matrix that was analyzed in order to generate a pairwise SNV matrix (S2 and S3 Datasets).

Accession number

The nucleotide sequence of the *ileS2*-encoding plasmid (p140355) from ST1-MRSA-IV-t127/t922 isolate M14/0355 has been submitted to GenBank (accession number: KY465818).

Supporting information

S1 Dataset. Pairwise SNV matrix of all 89 ST1-MRSA-IV isolates used to infer relatedness between isolates.
(XLSX)

S2 Dataset. Pairwise SNV matrix—swab (A). Pairwise SNV matrix of 13 colonies from a single swab (from the same patient from which isolate M13/0653 was recovered) used to determine the maximum intra-host variation of an ST1-MRSA-IV isolate and inform interpretation of the ST1 pairwise SNV matrix.
(XLSX)

S3 Dataset. Pairwise SNV matrix—swab (B). Pairwise SNV matrix of 13 colonies from a single swab (from the same patient from which isolate M15/0221 was recovered) used to determine the maximum intra-host variation of an ST1-MRSA-IV isolate and inform interpretation of the ST1 pairwise SNV matrix.
(XLSX)

S1 Table. Isolate information. Epidemiological, phenotypic and molecular characteristics of t127 and t922 methicillin-resistant *Staphylococcus aureus* isolates recovered from 17 hospitals, four other healthcare facilities and the community throughout Ireland between 2013 and 2016.
(PDF)

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Author Contributions

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Formal analysis: MRE PMK MS DCC ACS AL.

Funding acquisition: ACS.

Investigation: MRE PMK AL MS GIB.

Project administration: DCC ACS.

Resources: GIB MS HH BOC.

Supervision: DCC ACS.

Writing – original draft: MRE DCC ACS PMK.

Writing – review & editing: MRE PMK GIB BOC HH ACS DCC.

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Intra-Hospital, Inter-Hospital and Intercontinental Spread of ST78 MRSA From Two Neonatal Intensive Care Unit Outbreaks Established Using Whole-Genome Sequencing

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From 2009 to 2011 [transmission period (TP) 1] and 2014 to 2017 (TP2), two outbreaks involving community-associated clonal complex (CC) 88-MRSA *spa* types t186 and t786, respectively, occurred in the Neonatal Intensive Care Unit (NICU) of an Irish hospital (H1). This study investigated the relatedness of these isolates, their relationship to other CC88 MRSA from Ireland and their likely geographic origin, using whole-genome sequencing (WGS). All 28 CC88-MRSA isolates identified at the Irish National MRSA Reference Laboratory between 2009 and 2017 were investigated including 20 H1 patient isolates, two H1 isolates recovered from a single healthcare worker (HCW) 2 years apart, three patient isolates from a second hospital (H2) and one patient isolate from each of three different hospitals (H3, H4, and H5). All isolates underwent DNA microarray profiling. Thirteen international isolates with similar microarray profiles to at least one Irish isolate were selected from an extensive global database. All isolates underwent Illumina MiSeq WGS. The majority of Irish isolates (25/28; all H1 isolates, two H2 isolates and the H3 isolate) were identified as ST78-MRSA-IVa and formed a large cluster, exhibiting 1–71 pairwise allelic differences, in a whole-genome MLST-based minimum spanning tree (MST) involving all Irish isolates. A H1/H2, H1/H3, and H1 HCW/patient isolate pair each exhibited one allelic difference. The TP2 isolates were characterised by a different *spa* type and the loss of *hsdS*. The three remaining Irish isolates (from H2, H4, and H5) were identified as ST88-MRSA-IVa and dispersed at the opposite end of the MST, exhibiting 81–211 pairwise allelic differences. Core-genome MLST and sequence-based plasmid analysis revealed the recent shared ancestry of Irish and Australian ST78-MRSA-IVa, and of Irish and French/Egyptian ST88-MRSA-IVa. This study revealed the homogeneity of isolates recovered during two NICU outbreaks (despite *spa* type and *hsdS* carriage variances), HCW involvement in the outbreak transmission chain and the strain's spread

to two other Irish hospitals. The outbreak strain, CC88/ST78-MRSA-IVa, was likely imported from Australia, where it is prevalent. CC88/ST88-MRSA-IVa was also identified in Irish hospitals and was likely imported from Africa, where it is predominant, and/or a country with a large population of African descent.

Keywords: community-associated MRSA, NICU outbreak, ST78-MRSA-IVa, ST88-MRSA-IVa, whole-genome sequencing, core-genome MLST, whole-genome MLST, sequence-based plasmid analysis

INTRODUCTION

Staphylococcus aureus is both a prominent pathogen worldwide and an asymptomatic coloniser of the skin and mucosal membranes of humans and animals (Young et al., 2012). The success of *S. aureus* as a disease-causing agent is largely attributable to its ability to acquire and express a wide variety of virulence-associated and antimicrobial resistance genes, many of which can be transferred horizontally between cells on mobile genetic elements (Malachowa and Deleo, 2010). *Staphylococcus aureus* develops into methicillin-resistant *S. aureus* (MRSA) upon acquisition of *mecA* or *mecC*, located on the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) mobile genetic element, or the plasmid-located *mecB* gene, all of which encode alternate penicillin-binding proteins, conferring resistance to almost all β-lactam antibiotics (Katayama et al., 2000; Shore et al., 2011; Becker et al., 2018). Molecular epidemiological evidence indicates that MRSA have evolved independently from multiple lineages of methicillin-susceptible *S. aureus* in several environments and MRSA clonal groups are therefore often categorised as healthcare-associated (HCA), community-associated (CA) or livestock-associated (LA) (Lindsay, 2010). In recent years, however, it has become increasingly apparent that clonal groups are not limited to the environment in which they initially arose (Bal et al., 2016) and thus, this type of classification currently serves to define a strain's origin.

In-depth surveillance of MRSA, both locally and internationally, is essential in order to identify modes and routes of transmission and, ultimately, to develop effective strategies to prevent or limit transmission. Whole-genome sequence analysis provides optimal typing resolution, thus enabling the accurate determination of isolate relatedness. Over the past five years, both single nucleotide polymorphism (SNP) and allele-based approaches have been utilised during the analysis of bacterial next generation sequencing data. Initially, SNP analysis alone was generally applied to data sets (Price et al., 2013). While this method provides the highest discriminatory power available, a suitable reference genome is not always available and the wide variety of SNP filters for which parameters must be set can impede inter-study comparisons (Schürch et al., 2018). This approach is therefore ideally suited to the comparison of closely related isolates using study-specific reference genomes. In 2014, Leopold et al. built on traditional multilocus sequence typing (MLST) involving just seven loci, by using all 40 finished *S. aureus* genomes available in GenBank as of June 2013 to devise a core-genome (cg)MLST scheme, consisting of 1,861 loci (Leopold et al., 2014). This method provided a standardised tool for comparing the stable core-genome of *S. aureus* isolates. The cgMLST approach is therefore particularly well-suited to determining the relatedness of isolates recovered over a relatively long period of time and/or from disparate geographic regions, when the environmental conditions to which isolates have been recently exposed and thus, the accessory genome, are of lesser relevance. Recently, extended versions of this cgMLST scheme, which include accessory genome loci, have also been employed (Roisin et al., 2016; Sabat et al., 2017). This approach is typically referred to as whole-genome (wg)MLST and is suited to local outbreak investigations, during which, the comparison of entire genomes may be both appropriate and beneficial. Although there are no definitive cgMLST or wgMLST thresholds for assigning isolate relatedness, it has been suggested that a difference of $\leq$24 (core genome or whole-genome) alleles may be used as an approximate clonality guideline, however, the longer the time period over which the isolates were recovered, the higher the possibility of their exceeding this threshold (Schürch et al., 2018). Furthermore, sequence-based plasmid analysis may also be used during surveillance or outbreak investigations, although significant bioinformatics expertise is required to optimise short-read plasmid assemblies (Orlek et al., 2017).

Although whole-genome sequencing (WGS) offers optimal typing resolution, conventional molecular typing methods of moderate discriminatory power, such as *S. aureus* protein A (*spa*) typing, are commonly used during hospital outbreak investigations (Frénay et al., 1996). Furthermore, for retrospective compatibility with previous studies, MRSA isolates are still assigned to a MLST clonal complex (CC) and/or sequence type (ST) (Robinson and Enright, 2004) and to one of 13 main SCC*mec* types (Shore and Coleman, 2013; Wu et al., 2015; Baig et al., 2018). Sequence types share a CC if at least five of their seven traditional MLST alleles are identical to at least one other ST in the CC (Feil et al., 2003). The application of such typing techniques also facilitates global MRSA surveillance, allowing for the broader classification MRSA strains and their general categorisation as pandemic, endemic or sporadic. For example, the CA CC88 clones, ST78-MRSA-IV, and ST88-MRSA-IV/V, have both achieved endemic status in particular geographic regions (Monecke et al., 2011). ST78-MRSA-IV is a Panton-Valentine leukocidin (PVL)-negative clone that usually harbours the antimicrobial resistance genes *blaZ* and *erm*(A) (Monecke et al., 2011). First isolated in remote Western Australia in 1995 (O'Brien et al., 2009), ST78-MRSA-IV was the fourth most prevalent clone detected in Australia in 2012, accounting for 3.6% of all MRSA and 5.1% of all CA-MRSA (Coombs et al., 2014). Reports of this clone elsewhere, however, are lacking, with

the exception of a single isolate from Germany (Monecke et al., 2011). ST88 MRSA includes both PVL-positive and negative strains, often harbours the exfoliative toxin gene, *etA*, and is generally associated with SCC*mec* types IV and V, although ST88-MRSA-VI have been identified sporadically in Western Australia (Monecke et al., 2011). Although prevalent in the Far East (Ozaki et al., 2009; Zhang et al., 2009; Qiao et al., 2014) and predominant in Africa, where it accounts for 24.2–83.3% of all MRSA isolates (Breurec et al., 2011; Schaumburg et al., 2011), ST88-MRSA is sporadic in Europe and the Middle East (Monecke et al., 2012; RuŽičková et al., 2012; Vindel et al., 2014). CC88 MRSA have also been described in bulk tank milk and retail food (Parisi et al., 2016; Raji et al., 2016).

MRSA is endemic in Irish hospitals, where the HCA ST22-MRSA-IV strain has predominated since 2002 (Irish National Meticillin-Resistant *Staphylococcus aureus* Reference Laboratory, 2016). An extensive diversity of other MRSA strains and clones has also been identified in Ireland, several of which have been involved in significant hospital outbreaks, including CA strains ST772-MRSA-V, and ST1-MRSA-IV (Brennan et al., 2012; Kinnevey et al., 2014; Earls et al., 2017). Between 2009 and 2011, a suspected protracted outbreak occurred in the Neonatal Intensive Care Unit (NICU) of an Irish hospital (H1), in which seven CC88-associated MRSA-t186 isolates were identified during patient screening. Interestingly, a second suspected protracted outbreak involving a CC88-associated *spa* type occurred in the same NICU between 2014 and 2017, in which 15 MRSA-t786 screening isolates were recovered. Infants in NICUs are particularly vulnerable to serious infection and MRSA colonisation increases their risk of nosocomial infection (Geva et al., 2011). The present study used WGS to achieve three main objectives. Firstly, this study aimed to determine the relationship between isolates from both outbreaks and to identify putative transmission events. Secondly, the relatedness of the outbreak isolates to other CC88 MRSA identified in Ireland was investigated. Finally, considering that CC88 MRSA is not usually associated with Ireland or indeed other European countries, the present study sought to determine the relatedness of all CC88 MRSA recovered in Ireland to international comparator isolates.

MATERIALS AND METHODS

Isolates

All 28 CC88-MRSA isolates identified at the Irish National MRSA Reference Laboratory (NMRSARL) between January 2009 and February 2017 were investigated. Twenty-two of these isolates were recovered in the NICU of H1 during two suspected outbreaks which occurred from 2009 to 2011 (seven isolates from seven inpatients) and 2014 to 2017 (13 isolates from 13 inpatients and two isolates from one healthcare worker [HCW]), respectively. The outbreaks were initially suspected due to the number of isolates identified and their recovery in a single unit in the hospital. While this study included only one isolate per patient, two isolates (W19 and W28) were included from a single HCW, as they were recovered two years apart (in 2015 and 2017, respectively). The remaining six CC88 isolates from Ireland were recovered from inpatients of a second hospital [H2; $n = 3$ (2 NICU and one adult ward)] and in three additional hospitals (H3, $n = 1$; H4, $n = 1$; H5, $n = 1$). All Irish isolates were recovered during colonisation screening. Each isolate is represented by a letter, indicating recovery from either a patient (P) or healthcare worker (W), followed by a number, indicating the order in which the isolates were recovered (**Table 1**). Fifteen CC88-MRSA isolates recovered between 2001 and 2017 in Australia ($n = 4$), France ($n = 3$), Germany ($n = 5$), Tanzania ($n = 2$), and Egypt ($n = 1$) were also included in this study for comparison to the Irish isolates (**Table 1**). These international isolates were selected from the in-house strain collections at Abbott ([Alere Technologies GmbH] Jena, Germany) and the Institute for Medical Microbiology and Hygiene, Technical University of Dresden (Dresden, Germany) based on their genotypic similarity ($n = 13$) or dissimilarity ($n = 2$) to the Irish isolates from the present study (see DNA microarray section below for detailed description of international isolate selection). The strain collections include approximately 22,000 global *S. aureus* isolates, a selection of which have been previously reported (Monecke et al., 2008, 2011, 2016). Each international isolate is represented by a letter, indicating the country of recovery, followed by a number, indicating the order in which the isolates were recovered (**Table 1**).

Isolates were identified as *S. aureus* using the tube coagulase test or the Vitek MS Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry system (Vitek, bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Methicillin resistance was detected using 30-μg cefoxitin disks (Oxoid Ltd., Basingstoke, United Kingdom) in accordance with European Committee of Antimicrobial Susceptibility Testing methodology and interpretive criteria (European Committee on Antimicrobial Susceptibility Testing, 2017) or using the automated VITEK 1 or VITEK 2 systems (bioMérieux, Nuertingen, Germany). Isolates were stored at −80°C on individual Protect Bacterial Preservation System cryogenic beads (Technical Services Consultants Ltd., Heywood, United Kingdom).

Phenotypic Susceptibility Testing

The susceptibility of all isolates was determined against a panel of 19 antimicrobial agents, in addition to cefoxitin, by disk diffusion using European Committee of Antimicrobial Susceptibility Testing methodology (European Committee on Antimicrobial Susceptibility Testing, 2017), and previously described reference strains and interpretative criteria (McManus et al., 2015). The 19 agents tested were amikacin, ampicillin, chloramphenicol, clindamycin, ciprofloxacin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, neomycin, rifampicin, streptomycin, sulphonamide, tetracycline, tobramycin, trimethoprim, and vancomycin.

spa Typing

Genomic DNA for *spa* typing was extracted using the 6% InstaGene matrix solution, according to the manufacturer's instructions (BioRad, München, Germany). The variable X region in the *spa* gene of each isolate underwent PCR amplification using the primers and thermal cycling conditions

TABLE 1 | Epidemiological, phenotypic and genotypic details of the 43 CC88-MRSA isolates investigated.

Source	Isolate/patient no.	Month and/or year of recovery	Sequence type[a]	SCC*mec* type/subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
Ireland-H1	P1	Jan 2009	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P2	Apr 2009	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P3	Mar 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P4[f]	Nov 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P5[f]	Nov 2010	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P6	Aug 20/11	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	P7	Sep 2011	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, sak, scn, sec, sel*
	P10	Mar 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P11	Mar 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P13	Sep 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P14	Oct 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P15	Nov 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P18	Jan 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm(A), mecA, lukX, sak, scn, sec, sel*
	W19[g]	Jan 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P20	June 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P21	Sep 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P22	Oct 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P23	Nov 2015	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P24	Feb 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P26	June 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P27	June 2016	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	W28[g]	Feb 2017	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
Ireland-H2	P8	Aug 2013	88	IVa- CMFT503	t786	Ap, Cm, Fx, Tp	*blaZ, cadX, cat, dfrS1*, *mecA*, *hsdS, lukX, sak, chp, scn*
	P16	Nov 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
	P17	Dec 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
Ireland-H3	P12[h]	Sep 2014	78	IVa-MW2	t786	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, lukX, sak, scn, sec, sel*
Ireland-H4	P9	Sep 2013	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, etA*, *hsdS, lukX, sak, chp, scn*
Ireland-H5	P25	Mar 2016	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, etA*, *hsdS, lukX*, *sak, chp, scn*
Australia	A1	2001	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn*
	A2	2002	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	A3	2008	78	IVa-MW2	t186	Ap, Er, Fx, Tp	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
	A4	2008	78	IVa-MW2	t186	Ap, Fx, Tp	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn, sec, sel*
Egypt	E1[i]	2014	88	IVa- CMFT503	t13712	Ap, Fx, Tp	*blaZ cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*
France	F1	2002	88	IVa- CMFT503	t186	Ap, Er, Fx, Tp	*blaZ, cadX, dfrS1*, *erm*(C), *mecA, etA*, *hsdS, lukX, sak, chp, scn*
	F2	2002	88	IVa- CMFT503	t786	Ap, Er, Fx, Tp	*blaZ, cadX, dfrS1*, *erm*(C), *mecA, etA*, *hsdS, lukX, sak, chp, scn*
	F3	2002	88	IVa- CMFT503	t690	Ap, Fx, Te, Tp	*blaZ, cadX, dfrS1*, *mecA, tet*(K), *vga*(A), *etA*, *hsdS, lukX, sak, chp, scn*
Germany	G1	2008	78	IVa-MW2	t186	Ap, Er, Fx	*blaZ, cadX, erm*(A), *mecA, hsdS, lukX, sak, scn*
	G2	2016	88	IVa- CMFT503	t1028	Ap, Fx, Te, Tp	*blaZ, cadX, dfrS1*, *mecA, tet*(K), *hsdS, lukX, sak, chp, scn*
	G3	2017	88	IVa- CMFT503	t786	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*
	R1	2014	88	IVa-MW2	t17863	Ap, Er, Fx	*blaZ, cadX, erm*(C), *mecA, hsdS, lukF, lukS, lukX, sak, chp, scn*

(Continued)

TABLE 1 | Continued

Source	Isolate/patient no.	Month and/or year of recovery	Sequence type[a]	SCC*mec* type/subtype[b]	*spa* type[c]	Antimicrobial resistance profile[d]	Antimicrobial resistance and virulence-associated genes[e]
	R2	2017	88	IVa-MW2	t5041	Ap, Fx, Te, Cp	*blaZ, cadX, mecA, tet*(K), *hsdS, lukF, lukS, lukX, sak, chp, scn*
Tanzania	T1	2016	88	IVa- CMFT503	t690	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, hsdS, lukX, sak, chp, scn*
	T2	2016	88	IVa- CMFT503	t690	Ap, Fx, Tp	*blaZ, cadX, dfrS1*, *mecA, vga*(A), *etA*, *hsdS, lukX, sak, chp, scn*

[a] *Sequence types (STs) were assigned using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany). Allelic profiles: ST78, 22-1-14-23-12-53-31; ST88, 22-1-14-23-12-4-31.*

[b] *All SCCmec subtypes were detected using an SCCmec subtyping DNA microarray (Monecke et al., 2016). All Irish isolates underwent in silico analysis for predicted DNA SCCmec subtype microarray hybridisation profiles, while all other isolates underwent real-life DNA microarray analysis. Both SCCmec subtypes IVa-MW2 (GenBank accession: BA000033.2) and IVa-CMFT503 (GenBank accession: HF569113.1) have been described previously (Monecke et al., 2016).*

[c] *All H1 t186 isolates were involved in an outbreak between 2009 and 2011. All H1 t786 isolates were involved in an outbreak between 2014 and 2017. spa repeat successions: t186, 07-12-21-17-13-13-34-34-33-34; t786, 07-12-21-17-13-34-34-33-34; t690, 07-12-21-17-13-13-34-34-34-33-34; t1028, 07-34-33-34; t5041, 07-12-21-17-13-13-34-34-34-34-33-34; t13712, 07-12-21-17-13; t17863, 07-12-12-13-13-13-34-33-34.*

[d] *Antimicrobial resistance phenotypes were determined by testing the susceptibility of isolates to a panel of 20 antimicrobial agents including amikacin, ampicillin (Ap), cefoxitin (Fx), chloramphenicol (Cm), ciprofloxacin (Cp), clindamycin, erythromycin (Er), fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, neomycin, rifampicin, streptomycin, sulphonamide, tetracycline, tobramycin, trimethoprim (Tp) and vancomycin.*

[e] *All antimicrobial resistance and virulence-associated genes were detected using the S. aureus Genotyping Kit 2.0 system [Abbott (Alere Technologies GmbH), Jena, Germany]. All Irish isolates underwent in-silico analysis for predicted Genotyping Kit 2.0 DNA microarray hybridisation profiles, while all other isolates underwent real-life DNA microarray analysis.*

[f] *Isolates P4 and P5 were recovered from twins on the same day.*

[g] *Isolates W19 and W28 were recovered from the same healthcare worker two years apart.*

[h] *The patient from whom isolate P12 was recovered had been transferred from H1.*

[i] *Isolate E1 was recovered from a buffalo. All other isolates were recovered from humans.*

H, hospital; NA, not available.

described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet; http://www.seqnet.org). Resulting PCR products were purified using the GenElute PCR clean-up kit (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) and were sequenced commercially (Source Bioscience, Waterford, Ireland) using an ABI 3730xl Sanger sequencing platform. The Ridom StaphType software package version 1.5 (Ridom Gmbh, Würzburg, Germany) was used for *spa* sequence analysis and *spa* type assignment.

Genotyping and SCC*mec* Subtyping Using DNA Microarrays

The 15 international CC88 isolates underwent DNA microarray profiling using the *S. aureus* Genotyping Kit 2.0 system [Abbott (Alere Technologies GmbH)] and an additional SCC*mec* typing array (Monecke et al., 2016). The *S. aureus* Genotyping Kit 2.0 DNA microarray detects 333 target sequences, corresponding to approximately 170 different genes and their allelic variants, and encoding antimicrobial resistance and virulence-associated genes, species, and typing markers, and several SCC-associated marker genes. It assigns *S. aureus* isolates to MLST CCs/STs and SCC*mec* types. Detailed descriptions of the relevant genes, primers, and probes have been previously described (Monecke et al., 2008). The SCC*mec* typing array targets an additional 83 distinct sequences that are variably present in the SCC*mec* element and which form the basis of a previously described system that distinguishes between 61 different SCC*mec* subtypes (Monecke et al., 2016). Detailed descriptions of these SCC*mec*-linked genes/alleles and their corresponding primers and probes have been previously published (Monecke et al., 2016). Genomic DNA was extracted for both DNA microarray profiling and SCC*mec* array subtyping by enzymatic lysis using the *S. aureus* Genotyping Kit 2.0 [Abbott (Alere Technologies GmbH)] and the Qiagen DNeasy blood and tissue kit (Qiagen, West Sussex, United Kingdom). As all Irish isolates underwent WGS as part of the present study (see below), their genome sequences underwent *in silico S. aureus* Genotyping Kit 2.0 microarray profiling and SCC*mec* array subtyping. Virtual DNA array hybridisation patterns were generated whereby contigs were searched for probe binding sites and signal strength was dictated by the number of nucleotide mismatches, as previously described (Monecke et al., 2016). The CC, DNA microarray profile and SCC*mec* type/subtype was determined for each Irish isolate. Thirteen international isolates, with a similar DNA microarray profiles to at least one Irish isolate, were selected from the aforementioned global database, for WGS (**Table 1**). Two international "reference isolates" with dissimilar DNA microarray profiles to any of the Irish isolates were also selected for WGS, as controls (**Table 1**).

Whole-Genome Sequencing

All isolates underwent WGS using genomic DNA extracted as described above for DNA microarray profiling. DNA quality was assessed by UV absorbance using the NanoDrop spectrophotometer 2000 (ThermoFisher Scientific, Dublin, Ireland) and dilutions were performed using the Qubit Fluorometer 3.0 (ThermoFisher Scientific). The Nextera XT DNA Library Preparation Kit (Illumina, Eindhoven, The Netherlands) was used according to the manufacturer's instructions and libraries underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina). Libraries were scaled to exhibit at least 100x coverage and the quality of each sequencing run was assured following cluster density and Q30 assessment.

Whole-Genome Sequence Analysis

wgMLST and cgMLST

WGS data were analysed using the wgMLST scheme available in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) consisting of 3,904 *S. aureus* wgMLST loci (Roisin et al., 2016), including 1,861 cgMLST loci (Leopold et al., 2014). In order to ensure that all relevant alleles present were detected, two separate algorithms were used to generate a consensus whole-genome MLST profile for each isolate. The first method determined locus presence/absence and allelic identity using an assembly-free k-mer approach. The second, assembly-based method, used a BLAST approach to detect alleles on contigs assembled using the SPAdes software v3.7.1 (Bankevich et al., 2012) incorporated into BioNumerics. Default base correction parameters were applied and all contigs below 1000 bp were removed. The default settings were used for both the assembly-free and assembly-based algorithms. The quality of the sequence read sets, *de novo* assemblies, and assembly-free, and assembly-based allele calls, were assessed using the quality statistics window in BioNumerics and are detailed in Dataset S1. Traditional MLST sequence types were assigned using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany).

SNP Analysis

Isolates confirmed to be closely related following wgMLST subsequently underwent SNP analysis using a study-specific reference sequence. The SPAdes assembly of isolate P6 was chosen as the reference sequence due to both its central position in the wgMLST-based minimum spanning tree (MST) cluster and the high quality of its assembly. The BioNumerics Power Assembler mapping algorithm was used to create a consensus sequence for each sample and a pairwise distance matrix was generated. SNPs were called exclusively in positions shared by all samples. Only SNPs with at least 40x coverage were considered. Potentially indel-related SNPs, occurring within 12 bp of each other, were removed. Positions with ambiguous base calls and SNPs in repetitive regions were excluded.

Minimum Spanning Trees

Minimum spanning trees were constructed firstly, involving the Irish isolates exclusively and secondly, involving both the Irish and international isolates. For the Irish isolates, in order to identify the most appropriate analysis method, three separate MSTs were generated based on cgMLST, wgMLST or SNP data, and were examined in tandem with all available epidemiological and genotypic information. As the Irish and international isolates were recovered over 16 years and from disparate geographic regions, the construction of a cgMLST-based MST was deemed appropriate in this instance. All MSTs were generated using the permutation resampling function and default priority rule set in BioNumerics. The resampling support for each branch was examined to ensure the validity of the general MST structure.

Sequence-Based Plasmid Analysis

All isolate genomes underwent sequence-based plasmid analysis. Sequence read sets were assembled using SPAdes v3.11.1 (Bankevich et al., 2012) with a final kmer size of 127. All contigs under 500 bp and all conitgs with kmer coverage less than 3.0 were excluded. For each isolate, a scatter plot was generated depicting the GC content versus coverage for each contig. Putative plasmid-derived contigs were differentiated from chromosomal-derived contigs based on their elevated coverage, low GC content and the identity of their first and last 127 nucleotides, indicative of a circular replicon. All putative plasmid-derived contigs were blasted against The National Centre for Biotechnology Information database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and those which mapped to a known plasmid sequence in GenBank were considered to be confirmed plasmids. Any plasmid types present in the Irish isolates or in both the Irish and international isolates were identified. For each such plasmid type, a multi-sequence global alignment was constructed including all the newly identified plasmid sequences and the GenBank reference sequence, using MAFT v7.273 (Katoh et al., 2002).

RESULTS

Two Distinct Clusters of CC88-MRSA Isolates

The majority of Irish isolates were identified as *spa* type t786 ($n = 21$), while those remaining were identified as t186 ($n = 7$; **Table 1**). All seven t186 isolates were recovered during the first suspected H1 outbreak, while the t786 isolates were recovered either during the second suspected H1 outbreak ($n = 13$) or from one of the four alternative hospitals (H2-H5; **Table 1**). The majority of Irish isolates (25/28) were also identified as ST78-MRSA-IVa, harbouring an SCC*mec* type IVa element corresponding to that identified in the MW2 MRSA strain (GenBank accession: BA000033.2). This included all t186 isolates (7/7) and 18/21 t786 isolates. The remaining three t786 isolates were assigned to ST88, a single locus variant of ST78, and harboured an SCC*mec* type IVa element corresponding to that identified in the CMFT503 MRSA strain (GenBank accession: HF569113.1; **Table 1**). The presence of a hypothetical SCC*mec* terminus protein, Q9XB68, in the MW2-like SCC*mec* element and the presence of both an alternate SCC*mec* terminus protein, SCC*mec* terminus 01, and the LytTR domain DNA-binding regulator, Q931B7, in the CMFT503-like SCC*mec* element, distinguish the two SCC*mec* type IVa elements (Monecke et al., 2016).

Interestingly, a highly conserved gene within *S. aureus* lineages, the type I restriction modification system gene, *hsdS* (Waldron and Lindsay, 2006), was absent from all t786 ST78-MRSA-IVa isolates. It was subsequently noted that, according to the cgMLST-based MST, this unusual deletion had occurred twice within a relatively small population (Figure S1). Importantly, however, the wgMLST-based MST (**Figure 1**) indicated that the *hsdS* deletion occurred just once within this population. It was therefore concluded that the wgMLST-based tree likely depicted the evolutionary path of this strain more accurately than the cgMLST tree. To confirm/dispute this finding, a SNP-based MST was generated involving the relevant isolates (Figure S2). The structure of this tree was in agreement with that of

the wgMLST MST, confirming that the *hsdS* deletion likely occurred once during the strain's spread. Ultimately, considering that the application of SNP analysis was not appropriate for all 28 Irish isolates, the wgMLST-based MST was selected for detailed data interpretation. Therefore, any allelic distances stated herein between Irish isolates exclusively, refer to wgMLST loci, while those stated between Irish and international isolates, or between international isolates exclusively, refer to cgMLST loci, as outlined in the methods.

Whole-genome MLST further supported the differentiation of isolates as suggested by their SCC*mec* subtypes and traditional STs, grouping all 25 ST78-MRSA-IVa isolates into a large cluster at one end of a MST, while the three ST88-MRSA-IVa isolates dispersed at the opposite end of the tree. The ST78-MRSA-IVa and ST88-MRSA-IVa isolates differed by a minimum of 325 alleles and exhibited average pairwise allelic distances of 23.8 (min. = 1; max. = 71) and 140.7 (min. = 81; max. = 211), respectively (**Figure 1**).

ST78-MRSA-IVa

The 25 ST78-MRSA-IVa isolates which grouped into the large wgMLST-MST cluster were recovered at intervals of 0–30 months and included all H1 isolates, two H2 isolates (P16 and P17) and the H3 isolate (P12). All patients from whom ST78-MRSA-IVa isolates were recovered were neonates. The patient from whom the H3 isolate was recovered, had recently been transferred from H1. All ST78-MRSA-IVa isolates exhibited resistance to both ampicillin and erythromycin, and harboured the β-lactamase resistance gene, *blaZ*, the macrolide-lincosamide, and streptogramin B resistance gene, *erm*(A), the cadmium tolerance gene, *cadX*, the immune evasion complex (IEC) genes *sak*, and *scn* (IEC type E) and the enterotoxin genes *sec*, and *sel* (**Table 1**). Isolate P7 was the only isolate that lacked the leukocidin homologue, *lukX*. The maximum distance observed between any two directly linked nodes was 32 alleles, detected between t186 isolates, P1 and P7, which were recovered almost 3 years apart during suspected outbreak 1. All other directly linked isolates exhibited 1–19 allelic differences, significantly fewer than the recently proposed approximate clonality threshold of 24 alleles. This indicated a high degree of relatedness between the vast majority of directly linked isolates and a significant relationship between all isolates within the cluster network (**Figure 1**). Furthermore, there were no apparent sub-clusters dictated by *spa* type, suggesting that the "two outbreak strains" were homogeneous. Specifically, the branch that linked isolates P6 (t186) and P10 (t786) constituted the only direct link between the t786 and t186 isolates. However, this branch represented an allelic distance of 18, lower than both those of 19 and 32, each of which was observed elsewhere in the MST cluster. Interestingly, while the largely linear structure of the t186 isolates indicated that the outbreak strain was transmitted in a relatively sequential manner between 2009 and 2011, the highly branched network of t786 isolates suggested that a more complex transmission chain was established between 2014 and 2017 (**Figure 1**).

Isolates P4 and P5, which exhibited eight allelic differences, were recovered from twins on the same day, suggesting that

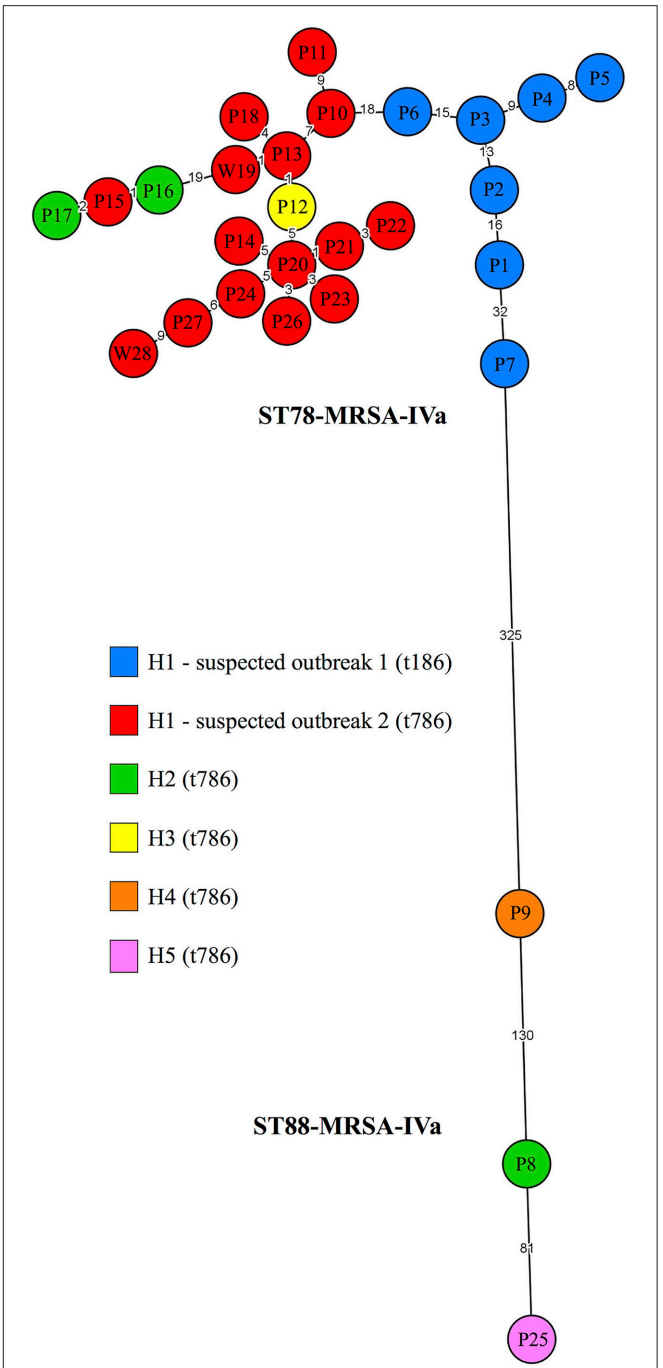

FIGURE 1 | A minimum spanning tree based on whole-genome multilocus sequence typing profiles of 28 CC88-MRSA isolates recovered in Irish hospitals between 2009 and 2017. Isolates are numbered in the order in which they were recovered. Isolate *spa* types are indicated by the colour legend. Suspected outbreak 1, involving seven t186 isolates, occurred in the NICU of H1 between 2009 and 2011. Suspected outbreak 2, involving 15 t786 isolates, occurred in the same NICU between 2014 and 2017. While one isolate was included per patient, two isolates (W19 and W28) recovered two years apart were included from a single healthcare worker. The remaining isolates were recovered from four different Irish hospitals. Isolates were identified as either ST78-MRSA-IVa or ST88-MRSA-IVa. Branch labels represent allelic distances. H, hospital; NICU, neonatal intensive care unit.

parallel or sequential acquisition may have occurred in this instance (**Figure 1**). One of the H2 isolates, P16, and a H1 isolate (P15) recovered 6 days before isolate P16, exhibited one allelic difference, strongly indicating that the outbreak strain spread between these two hospitals and suggesting transmission from the same source (**Figure 1**). Isolate P16 and the second H2 isolate (P17), which was recovered 44 days after isolate P16, exhibited three allelic differences, indicating further spread of this strain in H2 (**Figure 1**). Similarly, the only H3 isolate (P12) and a H1 isolate (P13) recovered 4 days after the H3 isolate, exhibited one allelic difference, clearly indicating that the outbreak strain spread from H1 to H3, and suggesting transmission from the same source (**Figure 1**). Interestingly, the two t786 isolates (W19 and W28) recovered from the same HCW two years apart exhibited 20 allelic differences, indicating that the strain had either altered over time *in vivo*, or that the HCW transiently carried different variants of the strain. Isolates W19 and W28 differed from the other t786 isolates by 1–21 (average:10.5) and 9–37 (average: 21.3) alleles, respectively, suggesting transmission of the outbreak strain between patients and the HCW (**Figure 1**).

ST88-MRSA-IVa

The three ST88-MRSA-IVa isolates, which exhibited an average of 140.7 pairwise allelic differences, included the final H2 isolate (P8), and the H4 (P9), and H5 (P25) isolates, all of which were t786 **(Table 1** and **Figure 1**). None of the patients from whom ST88-MRSA-IVa isolates were recovered were neonates (patients were aged 15 months, 25 years and 66 years). Two of the ST88-MRSA isolates (P8 and P9) were recovered from patients with names suggestive of a family connection to an African country. The phenotypic resistance profiles varied slightly amongst the ST88-MRSA-IVa isolates, all of which exhibited resistance to both ampicillin and trimethoprim, while isolate P8 exhibited chloramphenicol resistance (**Table 1**). The ST88-MRSA-IVa isolates also exhibited slightly differing genotypic profiles, all harbouring resistance genes *dfrSI*, encoding trimethoprim resistance, *blaZ,* and *cadX*, and the IEC genes *chp*, *sak,* and *scn* (IEC type B), while isolate P8 carried the chloramphenicol resistance gene, *cat*, and isolates P9 and P25 harboured *etA*, encoding exfoliative toxin A (**Table 1**). Considering these differences, the lack of epidemiological links and most importantly, the number of alleles by which they differed, these three isolates did not appear to be closely related.

Relatedness of Irish and International CC88 MRSA

Five of the 13 international CC88-MRSA isolates exhibiting similar array profiles to the Irish isolates were identified as ST78-MRSA-IVa-MW2. This included one German isolate (G1) recovered in 2008 and four Australian isolates, A1-A4, recovered in 2001, 2002, 2008 and 2008, respectively (**Table 1**). The remaining eight international isolates exhibiting similar array profiles to the Irish isolates were identified as ST88-MRSA-IVa-CMFT503. This included three French isolates (F1-3) recovered in 2002, two Tanzanian isolates (T1 and T2) recovered in 2016, one Egyptian isolate recovered in 2014 (E1) and two German isolates, G4 and G5, recovered in 2016 and 2017, respectively (**Table 1**). The two international reference isolates (R1 and R2 recovered in Germany in 2014 and 2017, respectively), were identified as ST88-MRSA-IVa-MW2. Following the construction of a cgMLST-based MST including all Irish and international isolates (Figure S3), international isolates R1 and R2 were excluded from further analysis as they failed to cluster with any other isolates, differing from their (shared) most closely related isolate (A1) by 189 and 198 alleles, respectively.

Irish and International ST78-MRSA-IVa Isolates

The five international ST78-MRSA-IVa isolates harboured the same resistance and virulence-associated genes as the Irish ST78-MRSA-IVa isolates (**Table 1**). The Irish and international ST78-MRSA-IVa isolates also exhibited very similar phenotypic susceptibility profiles, with both isolate groups exhibiting ampicillin and erythromycin resistance, while two international isolates (A3 and A4) also exhibited trimethoprim resistance. Interestingly, all international ST78-MRSA-IVa isolates were identified as t186 and none exhibited the *hsdS* deletion that characterised the Irish t786 ST78-MRSA-IVa isolates. Following the generation of a cgMLST-based MST including Irish and international isolates (**Figure 2)**, the international ST78-MRSA-IVa isolates grouped in relatively close proximity to the Irish ST78-MRSA-IVa cluster and exhibited an average pairwise allelic distance of 68.7 (min. = 36; max. = 105; **Figure 2**). Specifically, isolates A2-A4 and G1 all radiated independently from isolate A1, which was the only isolate that linked directly to the t186 side of the Irish cluster (**Figure 2**). Isolate A1 (recovered in 2001 in Australia) and its most closely related Irish isolate (P1; recovered in 2009) exhibited 60 allelic differences which, considering the disparate geographic regions and different time periods in which they were recovered, suggested a significant degree of relatedness between these two isolates.

All Irish and international ST78-MRSA-IVa isolates harboured a 21 kb plasmid encoding *blaZ* and *cadX*, corresponding to plasmid pWBG763 (GenBank accession number: GQ900467.1). Interestingly, however, the Irish ST78-MRSA-IVa isolates were characterised by a 100 bp deletion in this plasmid. All Irish and two Australian (A2 and A3) ST78-MRSA-IVa isolates also harboured a cryptic 2 kb plasmid corresponding to pWBG764 (GenBank accession number: GQ900468.1). Both plasmids pWBG763 and pWBG764 were originally sequenced from the same ST78-MRSA-IVa strain (WBG8366), which was recovered in remote Western-Australia in 1995.

Irish and International ST88-MRSA-IVa Isolates

The eight international ST88-MRSA-IVa isolates harboured similar resistance and virulence-associated genes to the Irish ST88-MRSA-IVa, all carrying *blaZ*, *cadX*, *dfrS1,* and the IEC genes *chp*, *sak* and *scn* (IEC type B), while exhibiting variable *etA* carriage (**Table 1**). However, two of the international ST88-MRSA-IVa isolates (F1 and F2) also harboured *erm*(C), encoding macrolide resistance, while two others (F3 and G2) harboured *tet*(K), encoding tetracycline resistance and a third pair (F3 and

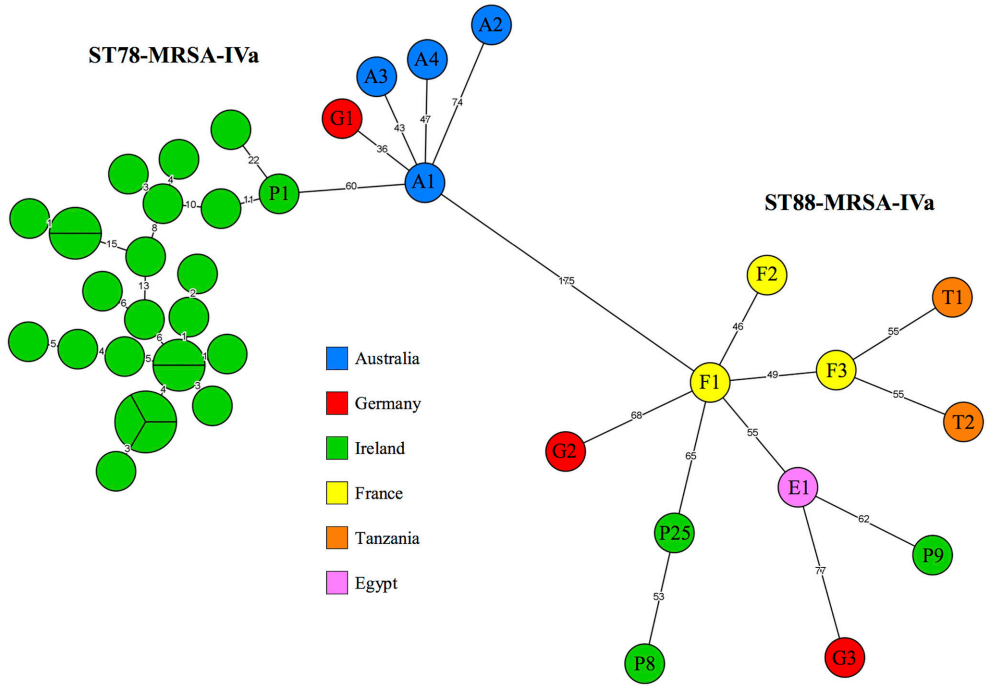

FIGURE 2 | A minimum spanning tree based on core-genome multilocus sequence typing profiles of 28 Irish and 13 international CC88-MRSA isolates recovered between 2001 and 2017. The countries in which the isolates were recovered are indicated by the colour legend. Isolates were identified as either ST78-MRSA-IVa or ST88-MRSA-IVa. Branch labels represent allelic distances.

T2) harboured *vga*(A), encoding streptogramin A resistance, none of which were present in the Irish isolates (**Table 1**). The Irish and international ST88-MRSA-IVa isolates also exhibited similar phenotypic susceptibility profiles, with both isolate groups exhibiting ampicillin and trimethoprim resistance, while two international ST88-MRSA-IVa isolates (F1 and F2) exhibited erythromycin resistance and two others (F3 and G2) exhibited tetracycline resistance, neither of which were observed in the Irish ST88-MRSA-IVa isolates. None of the international ST88-MRSA-IVa isolates exhibited chloramphenicol resistance, which was observed in one Irish ST88-MRSA-IVa isolate (P8). Isolate F1 was identified as t186, while the remaining international ST88-MRSA-IVa isolates were identified as t13712 ($n = 1$), t1786 ($n = 2$), t690 ($n = 3$) or t1028 ($n = 1$; **Table 1**). The eight international ST88-MRSA-IVa isolates formed a dispersed cluster with the three Irish ST88-MRSA-IVa isolates, in which an average pairwise allelic distance of 78.6 was observed (min. = 46; max. = 114; **Figure 2**). Irish isolates P25 and P9 differed from their most closely related international isolates (F1 and E1, respectively) by 65 and 62 alleles, respectively, indicative of shared ancestral genotypes. Irish isolate P8 and its most closely related international isolate (F1) exhibited 188 allelic differences, suggesting a lack of relatedness between the two isolates (**Figure 2**).

All Irish and international ST88-MRSA-IVa isolates harboured a 25 kb plasmid encoding *blaZ* and *cadX*, which corresponded to contig 10 (GenBank accession number: FMNJ01000010.1) of a previously published WGS project involving an MRSA isolate (GenBank accession number: FMNJ01000000.1) recovered in Tanzania in 2008. Irish ST88-MRSA-IVa isolate P8 also harboured a *cat*-encoding plasmid, which corresponded to contig 32 (GenBank accession number: LFNS01000032.1) of a previously published WGS project involving an ST3019 *S. aureus* isolate recovered in Ghana in 2013 (GenBank accession number: LFNS00000000.1).

DISCUSSION

The present study revealed the homogeneity of isolates involved in two outbreaks in the NICU of an Irish hospital. Although isolate *spa* types and recovery dates suggested that two different CC88-MRSA strains may have been involved in the outbreaks in this NICU, wgMLST revealed that these outbreaks were caused by the same CC88/ST78-MRSA-IVa strain, which spread within the ward during two separate transmission periods (transmission periods 1 and 2). This investigation highlighted both the involvement of a HCW in the outbreak transmission chain and the strain's spread to two other Irish hospitals. A cgMLST-based comparison with international comparator isolates revealed that the outbreak strain was most likely imported from Australia, where it is among the prevalent MRSA clones. This study also identified a second CC88-MRSA clone present in Irish hospitals, ST88-MRSA-IVa, which was likely imported from Africa, where it is predominant, and/or a country with a large population of African ethnic origin.

Transmission period 1 (TP1) involved the intermittent acquisition of the outbreak strain by seven NICU patients over a 32-month period. Interestingly, the topology of the MST indicated that all TP1 isolates, apart from P7, were acquired in a relatively sequential chain of transmission. While the topological characteristics of a phylogenetic tree can reveal invaluable relatedness and transmission details, both previously published studies and epidemiological data must also be drawn upon in order to gain meaningful insights into the dynamics of an outbreak. Notably, previous studies have indicated that patient-to-patient transmission is rare in adult intensive care units (Price et al., 2014; Wesley Long et al., 2014), a finding which is likely applicable to NICUs given the dependency of neonates on adults for mobility. Furthermore, it is unlikely that patients P1-P6 had overlapping stays (excluding twins, P4, and P5), considering the dates on which their isolates were recovered (**Table 1**). It may therefore be concluded that patient-to-patient transmission did not play a significant role in the outbreak during this period. Similarly, given that previous research suggests that *S. aureus* can survive a maximum of 90 days on hospital plastics and fabrics (Neely and Maley, 2000), and isolates P1-P6 were recovered at intervals of 4–11 months (**Table 1**), it is perhaps unlikely that patients P1-P6 acquired the outbreak strain directly from their environment without the involvement of another intermediary factor(s). Finally, previous studies have identified a role for HCWs in the transmission of MRSA to NICU patients (Geva et al., 2011; Brennan et al., 2012; Azarian et al., 2016). Considering these points, it appears highly likely that HCWs were involved in the spread of the outbreak strain during TP1, however, as routine HCW screening did not occur, their exact role cannot be definitively determined. Furthermore, these considerations, in combination with the topology of the MST, indicate that more than one vector was involved in spreading the outbreak strain during TP1. The data suggest two possible scenarios. Firstly, it is possible that a HCW constituted the primary outbreak source, originally seeding the outbreak strain in 2009, transmitting it to patient P1, and again in 2011, transmitting it to patient P7, while a different HCW initiated the strain's spread to patients P2-P6 (**Table 1**; **Figure 1**). Alternatively, the data suggest that patient P1 constituted the primary outbreak source and, while one HCW initiated transmission to patients P2-P6, a different HCW, who had also acquired the strain in 2009, eventually transmitted it to patient P7.

In Ireland, neonates are generally screened for MRSA upon admission into a NICU and weekly, thereafter (Irish Department of Health, 2013). Interestingly, however, the outbreak strain identified here was not detected between 2011 and 2014. It is unknown whether any staff changes or staff decolonisation occurred in H1 during this intervening period. Upon reappearing in 2014, the outbreak strain had undergone slight modifications which were detectable using conventional molecular epidemiological typing. Specifically, *spa* typing indicated that the *spa* gene had evolved from t186 to t786 (the latter of which is distinguishable from the former by the absence of one repeat unit), while DNA microarray profiling revealed an unusual *hsdS* deletion. It is highly likely that these alterations occurred locally, either while the strain resided *in-vivo* in a H1 HCW or during the strain's spread in the community, prior to reintroduction into the NICU.

Transmission period 2 (TP2) involved the acquisition of the outbreak strain, ST78-MRSA-IVa, by 20 patients and one HCW, over a 35-month period. Interestingly, the MST indicated that the vector from which patient P6 acquired the outbreak strain (during TP1), may have constituted the source of the outbreak at the beginning of TP2 (**Figure 1**). Furthermore, as observed during TP1, it appeared that more than one vector was involved in the spread of the outbreak strain during TP2. This was evident from the significant extension of three TP2 isolates (P15, P16, and P17, recovered in H1, H2, and H2, respectively) from the main body of the cluster in which isolates with both earlier and later recovery dates resided (**Figure 1**). In contrast to TP1, however, the TP2 isolates were recovered at intervals of 0–7 months suggesting that some TP2 patients may have had overlapping stays (**Table 1**). This circumstance may have contributed to the establishment of a more complex transmission chain during this time period. Although HCW screening is not mandatory in Ireland, it is indicated if transmission continues on a unit despite active control measures, if epidemiological aspects of an outbreak or strain are unusual, or if they suggest persistent MRSA carriage by staff (Irish Department of Health, 2013). This was likely the basis upon which HCW screening took place during TP2, the extent of which, is unknown. Importantly, an average pairwise distance of 10.5 (range: 1–21) between a H1 HCW isolate (W19) and the remaining TP2 isolates indicated that this HCW was likely directly involved in transmitting the outbreak strain to patients during this period. Similarly, a difference of one allele between both a H1/H2 (P15 and P16) and H1/H3 (P13 and P12) isolate pair, indicated that the outbreak strain spread to two additional hospitals. In the case of H3, it is highly likely that patient P12 acquired the outbreak strain in H1, before being transferred to H3. Similarly, although no known patient transfers occurred between H1 and H2, it is possible that a carrier who was not represented in the present study (i.e. a patient not screened during routine surveillance) was transferred from H1 to H2, during TP2. This is particularly feasible given the high frequency with which patients are transferred between Irish hospitals. However, as the employment of specialist healthcare staff by different hospitals is not uncommon in Ireland, it remains possible that the movement of staff facilitated the inter-hospital spread of this strain.

Genotypic data from both the present investigation and previously published studies were considered while determining the putative geographic origin(s) of the outbreak strain. Firstly, cgMLST indicated that the outbreak strain shared an ancestral genotype with an isolate recovered in Australia (**Figure 2**). Furthermore, a 21 kb *blaZ,* and *cadX*-encoding plasmid was detected in all Australian and Irish ST78-MRSA-IVa isolates, while a second cryptic 2 kb plasmid was detected in two Australian and all Irish ST78-MRSA-IVa isolates. Moreover, both of these plasmids were previously sequenced from the same Australian ST78-MRSA-IVa strain. Finally, ST78-MRSA-IVa is generally reported exclusively from Australia and the rate of travel between Australia and Ireland was consistently high in

the years preceding the study period (Australian Government Department of Immigration Citizenship, 2011). Considering these points, it is highly likely that the outbreak strain was imported from Australia, where it is commonly known as Western Australia MRSA-2 (Coombs et al., 2012). Interestingly, a 2012 study reported that while ST78-MRSA-IVa was the second most prevalent strain among HCWs in a Western Australian hospital, it was associated exclusively with persistent carriage (Verwer et al., 2012). This suggests, that even without constituting the predominant clone in a hospital setting, ST78-MRSA-IV colonisation may be particularly likely to persist, a phenomenon which may have contributed the continued spread of this strain to H1 NICU patients over the eight-year study period.

A second CC88-MRSA clone, ST88-MRSA-IVa, was also identified in Irish hospitals during the present study. In Ireland, patients with specific HCA-MRSA risk factors generally undergo screening for MRSA (Irish Department of Health, 2013). These guidelines likely formed the basis upon which three ST88-MRSA-IVa isolates were recovered from three different patients during the study period. However, considering both the lack of epidemiological links between these isolates and more importantly, the high number of alleles by which they differed, it was concluded that this strain was introduced into Irish hospitals on three separate occasions (**Figure 1**). Moreover, the non-neonatal status of these patients further supported the likelihood of their having acquired this strain (generally considered CA) outside of a healthcare setting, prior to admission.

Extensive genotypic, conventional epidemiological and previously published data were all considered while determining the region(s) from which ST88-MRSA-IVa was likely imported into Ireland. Firstly, cgMLST indicated that two Irish ST88-MRSA-IVa isolates, P9, and P25, shared ancestral genotypes with isolates recovered in Egypt and France, respectively (**Figure 2**). Furthermore, sequence-based plasmid analysis revealed that all Irish and international ST88-MRSA-IVa isolates harboured the same *blaZ* and *cadX*-encoding plasmid, previously sequenced from a Tanzanian MRSA isolate. This suggested that all ST88-MRSA-IVa investigated may have originated in relatively close geographic proximity. Moreover, an Irish ST88-MRSA-IVa isolate harboured an additional plasmid, previously sequenced from a Ghanaian *S. aureus* isolate. Secondly, ST88 MRSA has become increasingly associated with Africa in recent years and France is known to have a large population of African ethnic origin (Schaumburg et al., 2014; https://www.insee.fr/en/statistiques/1283070). Finally, two of the three patients from whom ST88-MRSA-IVa was recovered, had African names, suggesting they may have had family connections to an African country. Considering these points, it was concluded that ST88-MRSA-IVa was likely imported into Ireland from Africa and/or a country with a large population of African ethnic origin.

While WGS and DNA microarray profiling were successfully utilised to achieve the aims of the present study, two significant limitations, which often impede WGS-based studies, were also identified. Firstly, in the absence of universally accepted intra-host strain diversity guidelines, putative transmission events were not identified exhaustively. Notably, however, isolates recovered from twins (who generally share nursing care) on the same day, differed by eight alleles. Furthermore, isolates P10 and P11, which were recovered 3 days apart following a 30-month period in which the outbreak strain was not detected, differed by nine alleles. This suggests that an approximate intra-host strain diversity threshold of nine alleles may be applicable to the present study. Secondly, a lack of detailed epidemiological information limited the certainty with which conclusions could be drawn regarding the intricate dynamics of the H1 NICU outbreak, thus highlighting the importance of strong communicative links between healthcare facilities and research groups. However, despite the lack of detailed epidemiological information and the long period of time over which isolates were recovered, WGS provided robust and precise evidence of the occurrence of a protracted outbreak. Finally, this study highlighted the importance of considering all available epidemiological and genotypic information while selecting the whole-genome analysis approach best suited to the specific data set in question.

The present study revealed the HCW-facilitated spread of an Australian CA-MRSA strain, ST78-MRSA-IVa, in the NICU of an Irish maternity hospital over an eight-year period. Such findings indicate that further consideration of the role of HCWs in the transmission of MRSA in high-dependency units, such as NICUs, may be beneficial. This study also identified multiple introductions of an African CA-MRSA clone, ST88-MRSA-IVa, into Irish hospitals, suggesting that CA-MRSA risk factors should be considered during targeted patient screening. In a broader context, this study highlighted both the significance of travel in the spread of MRSA and the need for well-designed WGS-based studies that include in-depth epidemiological information in order to aid the establishment of data interpretation guidelines and thus, facilitate the real-time application of WGS in a clinical setting.

AUTHOR CONTRIBUTIONS

AS, DC, and GB conceived the study and provided the required resources. ME and TF performed the practical work. PS performed the *in silico* microarray and plasmid analysis. ME, AS, SM, RE and DC performed the remaining data analysis. ME and AS wrote the manuscript. DC, SM, RE, PS, and GB critically reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01485/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research paper

A novel multidrug-resistant PVL-negative CC1-MRSA-IV clone emerging in Ireland and Germany likely originated in South-Eastern Europe

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ABSTRACT

This study investigated the recent emergence of multidrug-resistant Panton-Valentine leukocidin (PVL)-negative CC1-MRSA-IV in Ireland and Germany.

Ten CC1-MSSA and 139 CC1-MRSA isolates recovered in Ireland between 2004 and 2017 were investigated. These were compared to 21 German CC1-MRSA, 10 Romanian CC1-MSSA, five Romanian CC1-MRSA and two UAE CC1-MRSA, which were selected from an extensive global database, based on similar DNA microarray profiles to the Irish isolates. All isolates subsequently underwent whole-genome sequencing, core-genome single nucleotide polymorphism (cgSNP) analysis and enhanced SCC*mec* subtyping.

Two PVL-negative clades (A and B1) were identified among four main clades. Clade A included 20 German isolates, 119 Irish isolates, and all Romanian MRSA and MSSA isolates, the latter of which differed from clade A MRSA by 47–130 cgSNPs. Eighty-six Irish clade A isolates formed a tight subclade (A1) exhibiting 0–49 pairwise cgSNPs, 80 of which harboured a 46 kb conjugative plasmid carrying both *ileS2*, encoding high-level mupirocin resistance, and *qacA,* encoding chlorhexidine resistance. The resistance genes *aadE*, *aphA3* and *sat* were detected in all clade A MRSA and the majority (8/10) of clade A MSSA isolates. None of the clade A isolates harboured any enterotoxin genes other than *seh*, which is universally present in CC1. Clade B1 included the remaining German isolate, 17 Irish isolates and the two UAE isolates, all of which corresponded to the Western Australia MRSA-1 (WA MRSA-1) clone based on genotypic characteristics. MRSA within clades A and B1 differed by 188 cgSNPs and clade-specific SCC*mec* characteristics were identified, indicating independent acquisition of the SCC*mec* element.

This study demonstrated the existence of a European PVL-negative CC1-MRSA-IV clone that is distinctly different from the well-defined PVL-negative CC1-MRSA-IV clone, WA MRSA-1. Furthermore, cgSNP analysis revealed that this newly defined clone may have originated in South-Eastern Europe, before spreading to both Ireland and Germany.

Abbreviations: CC, clonal complex; cgSNP, core-genome single nucleotide polymorphism; MGE, mobile genetic element; MDR, multidrug-resistant; NJT, neighbour-joining tree; ST, sequence type; SMRT, single-molecule real-time; SCC*mec*, staphylococcal chromosomal cassette *mec*; WA, Western Australia; wgMLST, whole-genome multilocus sequence typing; WGS, whole-genome sequencing

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1. Introduction

Staphylococcus aureus is both a major global pathogen and a prevalent commensal inhabitant of the skin and mucosal membranes of humans and animals (Aires-de-Sousa, 2017; Young et al., 2012). Approximately 25% of the *S. aureus* genome is composed of accessory genes, many of which are horizontally transferable between cells on mobile genetic elements (MGEs) such as plasmids, bacteriophages and pathogenicity islands (Carroll et al., 1995; Lindsay and Holden, 2004). The exact composition of the accessory genome is largely influenced by environmental factors and hence, *S. aureus* is capable of adapting to a wide variety of different hosts and stressful conditions (Lindsay and Holden, 2004; Malachowa and Deleo, 2010). Upon acquisition of the staphylococcal chromosomal cassette *mec* (SCC*mec*) MGE, which encodes either *mecA* or *mecC*, *S. aureus* develop into methicillin-resistant *S. aureus* (MRSA) (Katayama et al., 2000; Shore et al., 2011). MRSA exhibit resistance to almost all beta-lactam antibiotics and infection with multidrug-resistant (MDR) strains further limits treatment options (Assis et al., 2017; Hartman and Tomasz, 1984). Preventing the spread of MRSA is therefore of vital importance.

Global surveillance of MRSA is essential for the identification of international transmission routes and the subsequent development of effective infection prevention and control strategies (World Health Organisation, 2014). Surveillance investigations typically involve the categorisation of MRSA into presumptive clones (i.e. strains that have emerged from a single SCC*mec* acquisition event) and/or sub-clones. Traditionally, putative MRSA clones/sub-clones have been defined using conventional molecular typing techniques such as multilocus sequence typing (MLST), standard SCC*mec* typing, *spa* typing and PCRs for the detection of certain marker genes (Stefani et al., 2012; Coombs et al., 2012, 2014; Grundmann et al., 2014; Shore et al., 2014; Irish NMRSARL, 2016), including those encoding the Panton-Valentine leukocidin (PVL) (Panton and Valentine, 1932). Importantly, however, these methods involve the characterisation of only small subsections of the *S. aureus* genome and thus, their resolution is limited. More recently, whole-genome sequencing (WGS) has become increasingly widespread and the use of techniques such as single nucleotide polymorphism (SNP) analysis has enabled the quantitative comparison of strains with exceptionally high resolution (Earls et al., 2017; Kinnevey et al., 2016; Price et al., 2013). While WGS is generally considered the technical gold-standard during surveillance, a combination of DNA microarray profiling and enhanced SCC*mec* subtyping has also been used to accurately differentiate between clones (Earls et al., 2018; Monecke et al., 2011, 2016). As of 2017, approximately 300 different MRSA clones and 60 different SCC*mec* subtypes had been defined using these techniques (Monecke et al., 2016; *S. aureus* Genotyping Kit 2.0 Arraymate database (Abbott [Alere Technologies GmbH], Jena, Germany)). DNA microarray profiling and enhanced SCC*mec* subtyping can therefore be used to select relevant strains for WGS-based investigations.

To date, only one PVL-negative ST1-MRSA-IV clone has been well-defined in the literature (Monecke et al., 2011). Often referred to as Western Australia (WA) MRSA-1, this CC1 clone was first recovered in Australia in the late 1980s (Udo et al., 1993) and isolates indistinguishable from WA MRSA-1 by MLST, *spa* and array profiling have since been detected in the UAE, Egypt and Europe (Monecke et al., 2011). As suggested by its origin in the community, WA MRSA-1 is not typically associated with multidrug resistance. Specifically, this clone is generally associated with the penicillin resistance gene *blaZ*, the macrolide, lincosamide and streptogramin (MLS) resistance gene *erm*(C) and virulence-associated genes *sak*, *scn*, *sea*, *seh*, *sek* and *seq* (Coombs et al., 2011). The fusidic acid resistance gene, *fusC*, which is carried on a SCC element (SCC*fus*), is also common among WA MRSA-1. Furthermore, WA MRSA-57 and WA MRSA-45, the latter of which also harbours SCC*fus*, have been defined as sub-clones of WA MRSA-1 (Coombs et al., 2011). All three of these variants are typically associated with *spa* type t127 (Coombs et al., 2011). Interestingly, PVL-negative ST1-MRSA-IV-t127 have also been recovered from pigs, cattle, horses, rooks, companion animals and wild boars (Cuny et al., 2015; Loncaric et al., 2013, 2014; Porrero et al., 2013). Notably, however, these animal strains have not been investigated using WGS and their placement into a global context has not yet been established. A PVL-positive ST1-MRSA-IV clone known as USA400 has also been well-defined (Herold et al., 1998). Like WA MRSA-1/45/57, this clone is not typically associated with multidrug resistance.

A recent WGS-based study identified MDR PVL-negative CC1-MRSA-IV as the predominant CC1-MRSA clone among humans in Ireland (Earls et al., 2017). This study determined that a distinct variant of this specific CC1-MRSA-IV clone, which generally exhibited mupirocin resistance conferred by a conjugative *ileS2*-encoding plasmid (p140355), was responsible for a protracted hospital outbreak in Ireland (Earls et al., 2017). These findings were particularly alarming considering both the difficulty associated with treating MDR infections and the importance of mupirocin, which in many countries (including Ireland), is used in combination with chlorhexidine for nasal and body decolonisation, respectively (Irish Department of Health, 2013; Poovelikunnel et al., 2015). The aims of the present study relate to these recent findings. Firstly, this study investigated whether the MDR CC1-MRSA-IV clone identified in Ireland is present elsewhere. This involved comparing the whole-genome sequences and SCC*mec* elements of Irish MDR CC1-MRSA-IV and international isolates exhibiting similar genotypic characteristics to the Irish isolates, as determined by DNA microarray profiling. Secondly, considering that multidrug resistance is not typically associated with human PVL-negative CC1-MRSA-IV, this study investigated whether the MDR CC1-MRSA-IV clone identified in Ireland constitutes a sub-clone of WA MRSA-1/45/57 or a novel clone yet to be formally characterised. This involved (a) comparing the whole-genome sequences and SCC*mec* elements of MDR CC1-MRSA-IV and WA MRSA-1/45/57 and (b) comparing the whole-genome sequences of MDR CC1-MRSA-IV and MSSA isolates exhibiting similar genotypic characteristics to the MDR clone, as determined by DNA microarray profiling. Finally, considering the crucial role of mupirocin as a decolonising agent, this study aimed to further analyse the *ileS2*-encoding plasmid identified in the Irish outbreak variant in order to elucidate the factors driving its selection. To date, ten different *ileS2*-encoding plasmids have been described in staphylococci, six of which are members of the pSK41/pGO1 plasmid family and eight of which encode additional antimicrobial resistance genes (Ho et al., 2016; Pérez-Roth et al., 2010).

2. Materials and methods

2.1. Isolates

The present study included all 139 CC1-MRSA-IV isolates identified at the Irish National MRSA Reference Laboratory (NMRSARL) between 2007 and 2017 (Table 1). Eighty nine of these isolates (recovered between June 2013 and June 2016) underwent *spa* typing, DNA microarray profiling, WGS and MLST as part of the aforementioned study on a protracted hospital outbreak (Earls et al., 2017). During the present study, the remaining 50/139 isolates were identified as CC1-MRSA-IV based firstly, on their exhibiting *spa* types corresponding to CC1 and secondly, on their assignment to CC1-MRSA-IV following *in silico* DNA microarray profiling (see below). All available CC1 MSSA isolates (10 isolates recovered between 2004 and 2017) identified at the Irish NMRSARL based on *spa* typing, were included for comparison to the Irish CC1-MRSA-IV isolates (Table 1). The Irish *S. aureus* isolates included two PVL-positive MRSA and three PVL-positive MSSA isolates, which were included as potentially useful comparator isolates. Thirty-eight international *S. aureus* isolates were also investigated, including 21 MRSA isolates recovered in Germany between 2007 and 2018, five MRSA isolates recovered in Romania between 2010 and 2012, 10 MSSA isolates recovered in Romania between 2009 and 2012 and two MRSA

Table 1
Epidemiological and genotypic data associated with the 167 CC1-MRSA-IV isolates and 20 CC1-MSSA isolates investigated in the present study.

NJT position[a]	Country	MRSA/ MSSA	No. of isolates	Recovery period	Source(s)	Sequence type(s)[b]	*spa* type(s)[c]	SCC element subtypes
Subclade A1	Ireland	MRSA	86	2013–2017	10 hospitals/HCFs Community	ST1	t127 (n = 84) t922 (n = 2)	MW2-like SCC*mec* IVa with *dcs* insertion
Remaining clade A	Ireland	MRSA	32	2007–2017	11 hospitals/HCFs Community	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Germany	MRSA	20	2016–2018	3 hospitals Community	ST1(n = 19) ST4911 (n = 1)	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Romania	MRSA	5	2010–2012	1 hospital	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
		MSSA	10	2009–2012	1 hospital	ST1 (n = 8) ST4910 (n = 1) ST4912 (n = 1)	t127 (n = 8) t5633 (n = 1) t18248 (n = 1)	SCC-negative
Subclade B1	Ireland	MRSA	17	2012–2017	9 hospitals Community	ST1 (n = 15) ST4913 (n = 1) ST4914 (n = 1)	t127	MW2-like SCC*mec* IVa (n = 9) SCC*mec* IVa/SCC*fus*$_{476}$ (n = 8)
	UAE	MRSA	2	2009	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Germany	MRSA	1	2007	1 hospital	ST1	t127	MW2-like SCC*mec*
Remaining clade B	Ireland	MRSA	3	2014–2016	3 hospitals	ST1	t127	MW2-like SCC*mec* IVa (n = 1) SCC*mec* IVa/SCC*fus*$_{476}$ (n = 2)
		MSSA	6	2004–2017	4 hospitals	ST1	t127	SCC*fus*$_{476}$
Clade C	Ireland	MRSA	1	2016	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Ireland	MSSA	2	2013	2 hospitals	ST1	t127	SCC*fus*$_{476}$
Clade D	Ireland	MSSA	2	2017	1 hospital	ST1	t127	SCC-negative

Abbreviations: HCFs, healthcare facilities; NJT, neighbour-joining tree; ST, sequence type.
[a] Based on core-genome single nucleotide polymorphism analysis.
[b] Multilocus sequence typing was performed using Ridom SeqSphere+ version 4.1 (Ridom GmbH). Allelic profiles: ST1, 1-1-1-1-1-1-1; ST4910, 1-1-1-1-40-1-1; ST4911, 1-1-1-1-1-1-649; ST4912, 1-731-1-1-1-1-1; ST4913, 1-1-1-1-1-1-648; ST4914, 1-1-663-1-1-1-1.
[c] *spa* typing was performed either using Ridom SeqSphere+ version 4.1, or as previously described (Earls et al., 2018). *spa* repeat successions: t127, 07-23-21-16-34-33-13; t922, 07-23-21-16-33-13; t5633, 15-13; t18248, 07-23-21-16-34-33-20.

isolates recovered in from the UAE in 2009 (Table 1). These international isolates were selected from a *S. aureus* isolate microarray profile database including approximately 25,000 strains recovered from humans and animals worldwide. Further details relating to this global database and the rationale used to select the international isolates are described below. All available epidemiological data associated with the 187 (149 Irish and 38 international) isolates investigated are detailed in Table S1. Isolates underwent species identification and methicillin resistance detection as previously described (Earls et al., 2018). All isolates were stored at −80 °C on individual Protect Bacterial Preservation System cryogenic beads (Technical Services Consultants Ltd., Heywood, United Kingdom).

2.2. *spa* typing

For the Irish isolates, *spa* typing was performed with genomic DNA extracted using the InstaGene matrix solution (BioRad, München, Germany), according to the manufacturer's instructions. Amplification of the variable X region in the *spa* gene, PCR clean-up, DNA sequencing and *spa* type assignment were performed as previously described (Earls et al., 2018). All international isolates underwent *spa* typing using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany) following WGS (see below) (Bletz et al., 2015).

2.3. WGS

All isolates underwent WGS. Genomic DNA was extracted by enzymatic lysis using the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany) and the Qiagen DNeasy blood and tissue kit (Qiagen, West Sussex, United Kingdom). DNA quality was assessed and DNA dilutions were performed, as previously described (Earls et al., 2018). The Nextera XT DNA Library Preparation Kit (Illumina, Eindhoven, The Netherlands) was used according to the manufacturer's instructions and libraries underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina). Libraries were scaled to exhibit at least 50× coverage and the quality of each sequencing run was assured following cluster density and Q30 assessment. All reads were assembled into contigs using SPAdes v3.7.1 (Bankevich et al., 2012).

2.4. Genotyping and SCCmec subtyping using in silico DNA microarrays

The sequences of the hybridization probes utilised in *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany) DNA microarray and a previously described SCC*mec* subtyping array (Monecke et al., 2016) were mapped on the assembled genomes to generate hybridisation patterns comparable to real array hybridisation experiments, as previously described (Monecke et al., 2016). To compare the complement of antimicrobial and virulence-associated genes between groups of isolates, the two-tailed Fisher's exact test was performed using the GraphPad QuickCalcs website: https://www.graphpad.com/quickcalcs/contingency1/ (accessed July 2018). Results were considered statistically significant if $p < .05$. Specific SCC*mec*-related alleles of interest were compared using Clustal Omega (Sievers et al., 2011), while regions of interest within the SCC*mec* element were investigated using the publicly available National Centre for Biotechnology Information (NCBI) BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Details relating to the global S. aureus database

The database was generated using *in vitro* and/or *in silico* versions of the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany), comprising experimental datasets for ca. 25,000 MRSA and MSSA isolates, as well as *in silico* re-analyses of ca. 3000 published genome sequences. In addition, the aforementioned SCC*mec* subtyping array (Monecke et al., 2016) was applied experimentally to a subset of ca. 2000 MRSA isolates and *in silico*, to all the genome sequences analysed. The global database includes 385 CC1-MRSA-IV isolates recovered in Europe, Australia, the Middle East, the USA and New

Zealand. The vast majority (382/385; 99.2%) of these CC1-MRSA-IV isolates exhibit one of four main genotypic patterns, while those remaining exhibit unusual characteristics (such as SCC pseudoelements or composite SCC elements) and possibly represent sporadic strains or variants/mutants. Three of the four main CC1-MRSA-IV patterns each match the description of previously defined clones (Monecke et al., 2011). These clones are (i) PVL-negative strains with SCC*mec* IV or SCC*mec* IV + SCC*fus* elements, referred to as WA MRSA-1/45/57, (ii) the PVL-positive "USA400" strain and (iii) a PVL-positive strain with a SCC*mec* IV + SCC*fus* composite element that appears to be restricted to Australia and New Zealand. A fourth pattern can be defined that differs from any well-defined CC1-MRSA-IV clones in several markers (see Results). This pattern corresponds to CC1-MRSA-IV previously identified in Ireland (Earls et al., 2017) and Romania (Monecke et al., 2014). Hereafter, this fourth genotypic pattern is referred to as the "undefined" pattern or clone.

2.6. Selection of international isolates

Following *in silico* genotyping, the Irish isolates could be compared to those represented in the global database. The vast majority of Irish MRSA isolates investigated exhibited either the WA MRSA-1/45/57 or the undefined genotypic pattern. Representative (based on country/location of isolation, genotypic variations and/or date of recovery) selections of international MRSA isolates exhibiting each of these two genotypic patterns were selected from the global database for further analysis using WGS. Specifically, one German and two UAE CC1-MRSA-IV isolates exhibiting the WA MRSA-1/45/57 genotypic pattern, and a further nineteen German and five Romanian CC1-MRSA-IV isolates exhibiting the undefined genotypic pattern were selected. A representative selection of international MSSA isolates exhibiting the undefined genotypic pattern (minus the SCC*mec* genes) was also selected from the global database for further analysis using WGS. This included 10 Romanian CC1-MSSA isolates. Importantly, it was noted that under-resourced countries were, despite all efforts, often poorly represented in the database.

2.7. Neighbour-joining tree analysis

The WGS data were analysed using BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). All isolate genomes underwent whole-genome MLST (wgMLST) using both assembly-free and assembly-based algorithms, as previously described (Earls et al., 2018). This wgMLST scheme consists of 3904 wgMLST loci (Roisin et al., 2016), 1861 of which are core genes (Leopold et al., 2014). A multiple sequence alignment of the concatenated core genes was generated and a core-genome (cg) SNP analysis was performed. cgSNPs were called exclusively in positions shared by all samples. Only cgSNPs with at least 5× read coverage (including 1× coverage in each direction) were considered. Potentially indel-related cgSNPs, occurring within 12 bp of each other, were removed. Positions with ambiguous base calls and cgSNPs in repetitive regions were excluded. Two separate neighbour-joining trees (NJTs) were generated with permutation resampling (1000 replicates) based on the cgSNP analysis. The first NJT included all MRSA isolates only (Fig. S1). This tree was used to confirm the clonality of the isolates exhibiting the undefined genotypic pattern (Fig. S1). The second NJT included all MRSA and MSSA isolates (Fig. 1). This tree was used to determine whether any of the MSSA isolates investigated were closely related to the undefined clone. A distance matrix based on cgSNP differences was also generated. The quality statistics window in BioNumerics was used to assess the quality of the sequence read sets, de novo assemblies, and cgMLST allele calls. Traditional STs were assigned following MLST analysis using Ridom SeqSphere+ version 4.1 (Ridom GmbH).

2.8. Plasmid sequence analysis

A conjugative *ileS2*-encoding plasmid, p140355 (GenBank accession number: KY465818) was previously detected in 50 mupirocin-resistant CC1-MRSA-IV isolates from Ireland, all of which were also included in the present study (Earls et al., 2017). In this previous study, a single mupirocin-resistant CC1-MRSA-IV isolate underwent single-molecule real-time (SMRT) sequencing and the entire *ileS2*-encoding plasmid (p140355) was obtained on a single contig. The sequence reads of the remaining 49 mupirocin-resistant CC1-MRSA-IV isolates from that study were then aligned to the SMRT sequence. In the present study, the sequence reads of an additional 32 *ileS2*-encoding CC1-MRSA-IV isolates were mapped against the p140355 sequence using the Burrows-Wheeler aligner (BWA-mem; http://arxiv.org/abs/1303.3997). The Artemis sequence viewer (https://www.sanger.ac.uk/science/tools/artemis) was used to visually assess the mapping of reads. The genetic organisation of the SMRT-derived p140355 sequence was confirmed by PCR using either Go Taq DNA polymerase (Promega, Madison, Wisconsin, USA) or the Expand Long Template PCR system (Roche Products Ireland Limited, Dublin, Ireland), according to the manufacturers' instructions. The primers used are detailed in Table S2. SnapGene v4.1.9 was used to construct a genetic map of the plasmid (Fig. 2). All open reading frames (ORFs) comprising at least 30 codons were identified and annotated, if possible, using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This database was also used to compare the p140355 sequence with other plasmid sequences of relevance.

2.9. Accession numbers

The contigs of a representative isolate (clade A; A_01; undefined CC1-MRSA-IV genotypic pattern) were submitted to GenBank (accession number: RBVO00000000.1). The sequence read sets of all isolates investigated were submitted to the NCBI Sequence Read Archive database (accession number: PRJNA494507).

3. Results

3.1. Identification of four distinct clades using cgSNP analysis

A total of 2891 core-genome sequence positions exhibited polymorphisms that fulfilled all filtering criteria. Neighbour-joining tree analysis based on these 2891 positions revealed that the 187 isolates investigated grouped into one of four main clades (A, B, C and D; Fig. 1). Clade A included 143 MRSA isolates, 86 of which formed a tight subclade termed subclade A1, and 10 MSSA isolates. Clade B included 23 MRSA isolates, 20 of which formed a loose subclade termed subclade B1, and six MSSA isolates. Clade C included one MRSA and two MSSA isolates, while clade D included two MSSA isolates only.

The clade A MRSA isolates exhibited the undefined CC1-MRSA-IV genotypic pattern, while the subclade B1 isolates exhibited the WA MRSA-1/45/57 genotypic pattern. The clade A MRSA isolates were therefore characterised in detail and compared to the subclade B1 isolates. A single centrally-located subclade A1 isolate was selected as a subclade representative for all analyses involving clade A, while subclade A1 was examined independently. Hereafter, clade A is therefore described as including 58 MRSA isolates and 10 MSSA isolates. The remaining isolates in clade B and those in clades C and D were examined separately.

3.2. Identification of clade specific SCCmec characteristics

Clades A and B1 were differentiated by 171 cgSNPs, while clade A MRSA specifically were differentiated from clade B1 by 188 cgSNPs. All clade A MRSA isolates harboured a SCC*mec* type IVa element similar to that identified in the MW2 (USA400) MRSA strain (GenBank accession

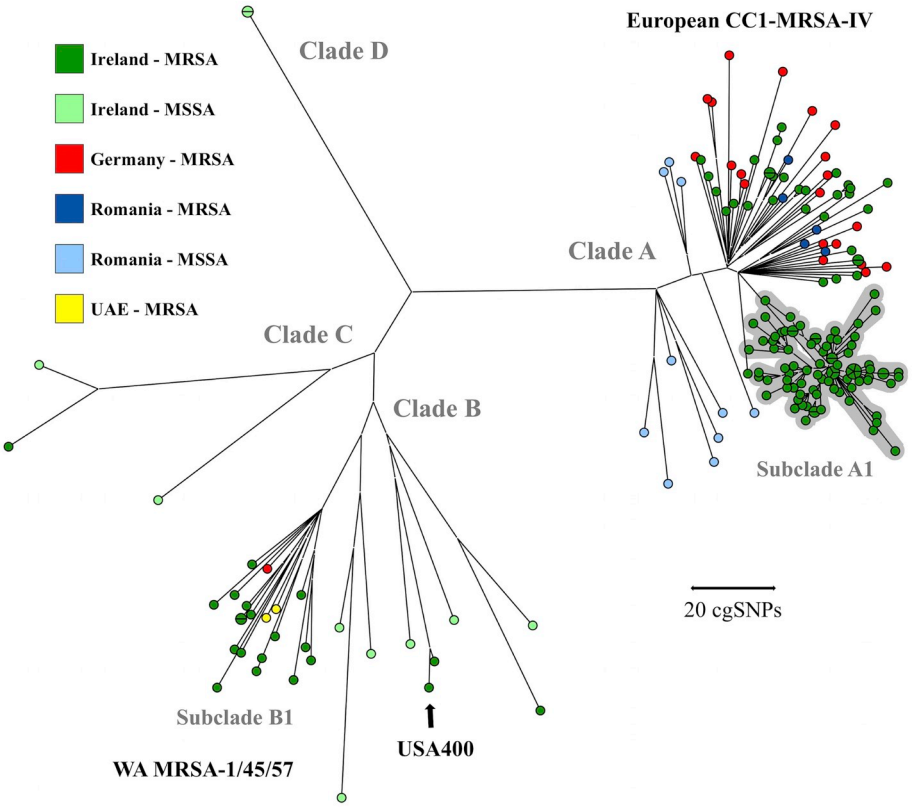

Fig. 1. A neighbour-joining tree based on a cgSNP analysis of 167 CC1-MRSA-IV and 20 CC1-MSSA isolates. The countries in which the isolates were recovered are indicated in the colour legend. Subclade A1 is shaded in grey. All branches yielded 100% permutation resampling support. The WA MRSA-1/45/57 isolates and the Irish clade B isolate marked as USA400 were identified based on genotypic characteristics. Abbreviation: cgSNPs, core-genome single nucleotide polymorphisms.

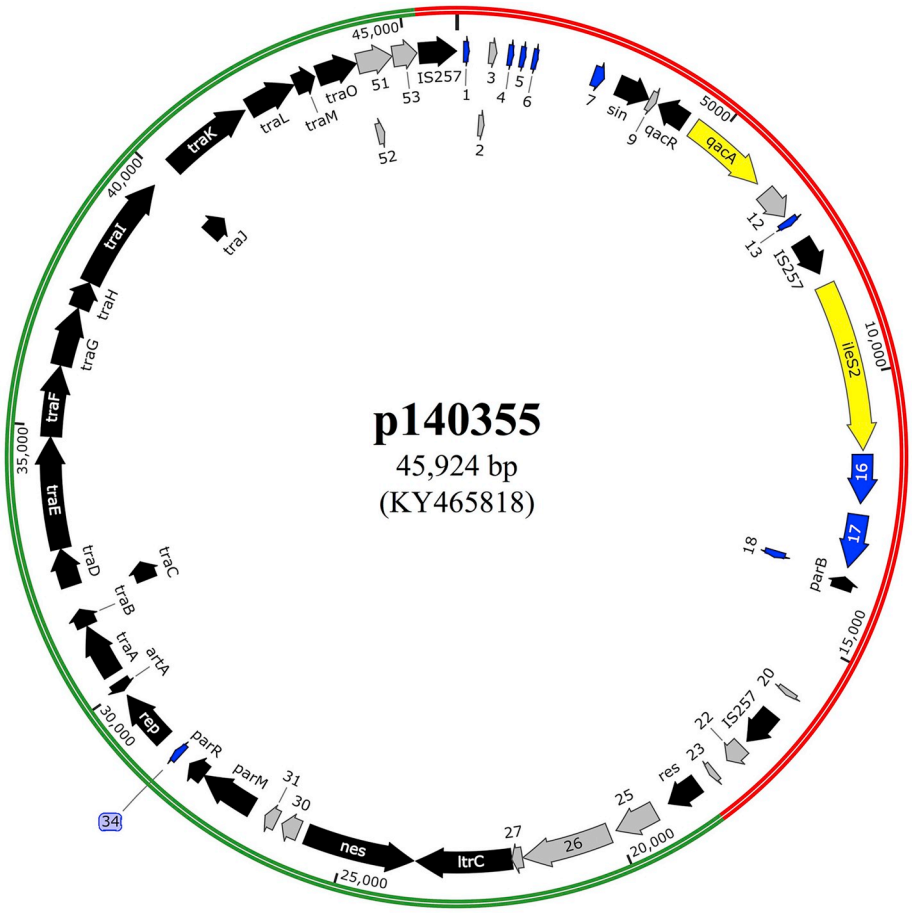

Fig. 2. Genetic map of *S. aureus* multi-resistance plasmid, p140355 (GenBank accession number: KY465818.1). The plasmid backbone is shown in green, while the accessory region is depicted in red. All known genes are shown in black except for the antimicrobial resistance genes, which are highlighted in yellow. Genes which have undergone National Centre for Biotechnology Information prediction are shown in blue: 1, MobA/MobL family protein; 4, Fst family toxin; 5, quinone reductase; 6, quinone reductase; 7, MarR family transcriptional regulator; 13, haloacid dehalogenase-like hydrolase family protein; 16, fructosamine kinase family protein; 17, major facilitator superfamily protein; 18, DDE transposase superfamily protein; 34, XRE family transcriptional regulator. Genes encoding hypothetical proteins are shown in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number: BA000033.2). In contrast to MW2-like SCC*mec* IVa, however, the clade A SCC*mec* element harboured a 4710 nucleotide insertion in the downstream constant segment (*dcs*) adjacent to *orfX*. This sequence encodes five different hypothetical proteins and corresponds to nucleotide positions 280,690–285,400 of GenBank entry RBVO000005.1. This gene cluster has also been detected in the SCC*mec* elements of several *S. aureus* (e.g. GenBank accession number: CP007672.1) and *Staphylococcus epidermidis* (e.g. LT571449.1) strains and one *Staphylococcus capitis* strain (CP007601.1). The subclade B1 isolates harboured MW2-like SCC*mec* IVa (without the *dcs* insertion). Half (10/20) of the subclade B1 isolates also carried a SCC*fus* element that included the *tirS* marker gene, *ccrA1* and *ccrB1* (i.e. SCC*fus*$_{476}$, as described in GenBank entry BX571857.1).

All clade A MRSA isolates harboured the same allelic variants of the cassette chromosome recombinase genes, *ccrA2* (1350 bp) and *ccrB2* (1629 bp). All subclade B1 isolates harboured the same *ccrA2* allele and the vast majority (16/20) harboured the same *ccrB2* allele, while those remaining harboured an allele which differed from the predominant *ccrB2* allele by 1 SNP. The *ccrA2* alleles in the SCC*mec* elements of clades A and B1 differed by 23 SNPs. The *ccrB2* allele in the clade A SCC*mec* element differed from the predominant *ccrB2* allele in the subclade B1 SCC*mec* element by 51 SNPs.

3.3. Clade A MRSA

The 58 clade A MRSA isolates were recovered in Ireland (n = 33), Germany (n = 20) and Romania (n = 5), and exhibited 0–109 (average 62.6, standard deviation [SD] 13.8) pairwise cgSNPs (Fig. 1; Table 1). All isolates were identified as ST1-MRSA-IVa-t127, with the exception of a single ST4911-MRSA-IVa-t127 isolate from Germany (Table 1). The 33 Irish MRSA isolates were recovered in 11 different hospitals/healthcare facilities (HCFs) and the community between 2007 and 2017 (Table 1) (Earls et al., 2017). Notably, the first and second clade A MRSA isolates identified in Ireland (which differed by 52 cgSNPs) were recovered in the community in 2007 and 2008, respectively, while the third such isolate was recovered in a hospital in 2012 (Table S1). The German isolates included 1/5 isolates (i.e. one of five isolates in the global database exhibiting the undefined genotypic pattern) recovered in a hospital in the Saxon city of Dresden in 2016, and 19/37 isolates recovered between 2016 and 2018 in two different hospitals and the community in the Bavarian city of Regensburg. The Romanian isolates included 5/40 isolates recovered in a hospital in the North-Eastern city of Iaşi between 2008 and 2012. Interestingly, no phylogenetic subgrouping of isolates recovered in the same country was observed (Fig. 1). Indeed, the Irish isolates exhibited 1–102 (average 69.0, SD 14.0) pairwise cgSNPs, the German isolates exhibited 12–115 (average 79.0, SD 16.2) pairwise cgSNPs and the Romanian isolates exhibited 53–71 (average 59.0, SD 6.8) pairwise cgSNPs.

The MRSA isolates within clade A harboured a broader range of antimicrobial resistance genes than those in clade B (Table 2). Specifically, *aphA3*, encoding neomycin and kanamycin resistance, *aadE*, encoding aminoglycoside resistance and *sat*, encoding streptothricin resistance, were detected in all clade A MRSA but were universally absent from clade B. Furthermore, *tet*(K), encoding tetracycline resistance, and *erm*(C) were more common in MRSA in clade A than in clade B1 (Table 2). The majority (47/58) of clade A MRSA isolates harboured the immune evasion cluster (IEC) type E (*sak* and *scn*), while those remaining harboured an undisrupted *hlb* gene. None of the clade A MRSA isolates harboured any enterotoxin genes other than *seh*, which is universally present in CC1.

3.4. Subclade A1

The 86 Irish MRSA isolates within subclade A1 exhibited 0–49 (average 17.4, SD 8.6) pairwise cgSNPs, and were recovered in 10 different hospitals/HCFs and the community, between 2013 and 2017 (Table 1) (Earls et al., 2017). The vast majority (84/86) of these isolates were characterised as ST1-MRSA-IVa-t127, while those remaining were identified as ST1-MRSA-IVa-t922. The majority (60/86; 70%) of subclade A1 isolates were recovered in a single hospital (Table S1).

The *ileS2* gene was markedly prevalent among subclade A1 isolates, but absent from (non-subclade A1) clade A MRSA (Table 2). Almost all (80/82) *ileS2*-encoding isolates also harboured the general efflux pump-encoding gene, *qacA*, which confers resistance to chlorhexidine among other compounds (Table 2). A ~7 kb insertion encoding *qacA*, was identified upstream of the *ileS2* region (Fig. 2). The only two other *ileS2* and *qacA*-encoding plasmid sequences (GenBank accessions numbers: KU882683 and KU882684) in the NCBI database correspond to plasmids unrelated to p140355 (50% and 24% query cover, respectively) that were previously recovered from *Staphylococcus lugdunensis*. The p140355 plasmid was characterised as a 45,924 bp circular plasmid of the pSK41/pGO1 family, exhibiting a GC content of 28.6% (Fig. 2). The plasmid backbone accounted for approximately 27.5 kb of the p140355 sequence, while the remaining 18.5 kb comprised the accessory region (Fig. 2). A total of 54 ORFs were identified, 29 of which encode known genes, 10 of which have undergone NCBI prediction and 15 of which encode hypothetical proteins (Fig. 2). The sequence reads of the 80 *ileS2* and *qacA*-encoding isolates mapped well to the p140355 sequence (Earls et al., 2017). Correspondingly, the sequence reads of the two *ileS2*-encoding isolates which lacked *qacA* (A1_01 and A1_61) failed to map to the *qacA* region but mapped well to the remainder of the plasmid sequence. The *tet*(K) gene and IEC genes, *sak* and *scn*, were also more common in subclade A1 than in clade A MRSA (Table 2).

3.5. Clade A MSSA

Considering that clade A included MRSA isolates recovered in Ireland and Romania as early as 2007 and 2009, respectively (Fig. 1; Table 1), CC1 MSSA from both of these countries were considered potential precursors to clade A MRSA. Forty Romanian CC1-MSSA isolates exhibiting the undefined CC1-MRSA-IV genotypic pattern (excluding genes typically located in SCC*mec*) were identified in the global database. Ten of these isolates were selected for WGS and subsequent cgSNP analysis. They grouped into clade A, differing from the MRSA isolates within this clade by 47–130 cgSNPs (Fig. 1). The Romanian MSSA isolates were recovered in the same hospital as the Romanian ST1-MRSA-IV isolates. The majority (8/10) of Romanian MSSA isolates were characterised as ST1 MSSA, while two isolates were identified as ST4910 and ST4912 (Table 1). Similarly, the majority (8/10) of Romanian MSSA isolates were identified as *spa* type t127, while the ST4910 isolate was identified as *spa* type t5633 and one ST1 isolate was assigned *spa* type t18248 (Table 1). Similar to clade A MRSA, 8/10 clade A MSSA isolates carried *aphA3*, *aadE* and *sat*. The *erm*(C) gene was also detected in 8/10 isolates, while 9/10 isolates harboured *tet*(K). Furthermore, clade A MSSA did not carry any enterotoxin genes other than *seh*, and either harboured IEC type E (8/10) or lacked IEC associated genes (2/10). Importantly, none of the 10 Irish MSSA isolates investigated grouped in close proximity to the clade A MRSA isolates (Fig. 1).

3.6. Subclade B1 (MRSA only)

The subclade B1 isolates were recovered in Ireland (n = 17), the UAE (n = 2) and Germany (n = 1), and differed by 0–116 (average 77.9, SD 17.0) pairwise cgSNPs. All subclade B1 isolates were identified as t127 (Table 1). The Irish isolates were recovered in nine different hospitals and the community between 2012 and 2017 (Table 1). Eight of the Irish isolates were identified as ST1-MRSA-IVa/SCC*fus*$_{476}$, while seven were identified as ST1-MRSA-IVa and the remaining two were characterised as ST4913-MRSA-IVa and ST4914-MRSA-IVa. The UAE isolates included 2/4 isolates recovered in a hospital in Abu Dhabi in 2009, both of which were characterised as ST1-MRSA-IVa/SCC*fus*$_{476}$.

Table 2
Differences in resistance and virulence-associated gene carriage between CC1-MRSA-IV in clade A and subclade B1, and in subclade A1 and the remainder of clade A.

Gene	Clade A[a]		Subclade B1[a]		*p* value[a]	Subclade A1[a]		Remaining clade A		*p* value[a]
	n	%	*n*	%		*n*	%	*n*	%	
Resistance										
aadD	0	0	**3**	**15**	**0.02**	1	1	0	0	1
aadE	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
aacA-aphD	2	3	0	0	1	0	0	2	4	0.16
aphA3	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
blaZ	58	100	20	100	1	86	100	57	100	1
erm(A)	0	0	**4**	**20**	**0.0034**	0	0	0	0	NA
erm(C)	**57**	**98**	3	15	**< 0.0001**	86	100	57	98	1
fusB	0	0	0	0	NA	5	6	0	0	0.16
fusC	0	0	**10**	**50**	**< 0.0001**	0	0	0	0	NA
ileS2	1	2	0	0	1	**82**	**95**	0	0	**< 0.0001**
lnu(A)	0	0	0	0	NA	2	2	0	0	0.52
mecA	58	100	20	100	1	86	100	57	100	1
qacA	2	3	0	0	1	**80**	**93**	1	2	**< 0.0001**
sat	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
tet(K)	**53**	**91**	0	0	**< 0.0001**	**85**	**99**	52	91	**0.04**
Virulence										
hlb[b]	11	19	0	0	0.06	2	2	**11**	**19**	**0.002**
sak	47	81	20	100	0.06	**84**	**98**	46	81	**0.002**
scn	47	81	20	100	0.06	**84**	**98**	46	81	**0.002**
sea	0	0	**19**	**95**	**< 0.0001**	0	0	0	0	NA
seh	58	100	20	100	1	86	100	57	100	1
sek	0	0	**19**	**95**	**< 0.0001**	0	0	0	0	NA
seq	0	0	**19**	**95**	**< 0.0001**	1	1	0	0	1

[a] Statistically significant results are shown in bold.
[b] Undisrupted-*hlb*.

The German isolate represented a group of four isolates recovered in the aforementioned Dresden hospital in 2007 and was identified as ST1-MRSA-IVa. A large group of Australian isolates ($n = 46$; recovered between 2001 and 2009) exhibiting WA MRSA-1/45/57 genotypic patterns were also identified in the global database. However, these Australian isolates did not undergo WGS as part of this study.

Apart from *fusC*, *aadD* (encoding aminoglycoside resistance) and *erm*(A) (encoding MLS resistance) were the only resistance genes that were more common in subclade B1 than in clade A MRSA (Table 2). However, the subclade B1 isolates harboured a wider range of virulence-associated genes than clade A MRSA (Table 2). Specifically, in addition to the CC1-associated *seh* gene, the enterotoxin genes *sea*, *sek* and *seq* were significantly common in clade B (Table 2). The majority (19/20) of subclade B1 isolates harboured IEC type D (*sea*, *sak* and *scn*), while IEC type E (*sak* and *scn*) was detected in a single instance.

3.7. Remaining isolates

All remaining isolates were recovered in Ireland (Fig. 1). The nine remaining (non-subclade B1) clade B isolates differed from those in subclade B1 by 83–158 cgSNPs. These isolates included two closely related (10 cgSNPs) PVL-positive ST1-MRSA-IVa-t127 isolates, one of which was identified as USA400 based on its genotypic pattern and the other of which harboured SCC*fus*$_{476}$ (Fig. 1). Clade B also included six ST1-MSSA-t127 isolates, all of which harboured SCC*fus*$_{476}$ and half (3/6) of which were PVL-positive. Clade C included one ST1-MRSA-IVa-t127 and two ST1-MSSA-t127 isolates, all three of which were PVL-negative and harboured SCC*fus*$_{476}$. The clade C MRSA isolate was relatively closely related (38 cgSNPs) to one of the MSSA isolates, while the remaining clade C MSSA isolate differed from its closest neighbour by 119 cgSNPs. Clade D included two PVL- and SCC-negative ST1-MSSA-t127 isolates which were indistinguishable using cgSNP analysis.

4. Discussion

This study confirmed the existence of a previously undefined PVL-negative CC1-MRSA-IV clone that may have emerged in Romania or neighbouring regions and has become prevalent in both Ireland and Bavaria, Germany. This clone is distinctly different to the well-characterised PVL-negative CC1-MRSA-IV clone known as WA MRSA-1 (and closely related WA MRSA-57 and WA MRSA-45), which was first identified in Australia. Although indistinguishable using a combination of MLST, SCC*mec* typing and *spa* typing, cgSNP and SCC*mec* analysis revealed a clear distinction between these two clones. Both clones were identified among PVL-negative CC1-MRSA-IV recovered in Ireland. A mupirocin-resistant variant of the newly defined European CC1-MRSA-IV clone harbouring a conjugative *ileS2*-encoding plasmid first came to notice following a protracted hospital outbreak in Ireland (Earls et al., 2017). In the present study, this plasmid was shown to harbour both *ileS2* and *qacA*, encoding resistance to mupirocin and chlorhexidine, respectively, which are commonly recommended as a treatment combination for MRSA decolonisation (Poovelikunnel et al., 2015).

Analysis of CC1-MRSA-IV isolates from Ireland, Germany, Romania and the UAE identified two main PVL-negative CC1-MRSA-IV clades (A and B1) which were differentiated by 188 cgSNPs (Fig. 1). Clade-specific SCC*mec* characteristics identified in the *ccr* genes and *dcs* indicated that the clones represented by these clades likely evolved from MSSA following separate SCC*mec* acquisition events. Furthermore, a potential marker sequence of the clone represented by clade A was identified as an insertion within *dcs*. Genotypic evidence strongly suggests that clade B1 represents the WA MRSA-1/45/57 clone. Indeed, clade B1 isolates generally harboured *blaZ*, *sak*, *scn*, *sea*, *seh*, *sek* and *seq* (Table 2), all of which are characteristic features associated with WA MRSA-1/57/45 (Coombs et al., 2011). Interestingly, the clade B1 isolates which carried SCC*fus*$_{476}$ (i.e. the isolates matching the WA-45 description) were interspersed between those which did not harbour SCC*fus*$_{476}$ (i.e. those matching the WA-57 description). Furthermore, a high degree of relatedness (10 cgSNPs) was noted between two PVL-positive MRSA isolates in clade B, only one of which carried SCC*fus*$_{476}$. These observations bring into question the stability of the SCC*fus*$_{476}$ element, although further studies are warranted. There is limited information on fusidic acid consumption in Ireland and Germany, however, it is often

used in the community in Europe (Dobie and Gray, 2004; Mason and Howard, 2004). In contrast to clade B1, clade A isolates exhibited genotypic characteristics which differed substantially from those associated with WA MRSA-1/45/57. Specifically, clade A isolates generally harboured *aphA3*, *aadE*, *sat* and *tet*(K), none of which are associated with WA MRSA-1/45/57, and lacked *sea*, *sek* and *seq*, all of which are associated with WA MRSA-1/45/57 (Coombs et al., 2011) (Table 2). Notably, the genotypic characteristics exhibited by clade A isolates did not correspond with USA400 either (Côrtes et al., 2017), suggesting it unlikely that the clade A clone derived from USA400 by loss of the genes encoding PVL. Indeed, microarray genotyping identified one of the clade B isolates as USA400 (Fig. 1).

A 2014 study used microarray genotyping to identify PVL-negative CC1-MRSA-IV as the predominant MRSA clonal group in a Romanian hospital between 2008 and 2012 (Monecke et al., 2014). Distinct genotypic differences were noted between this CC1-MRSA-IV clone and WA MRSA-1/45/57 (Monecke et al., 2014). It was hypothesised in the Romanian study that this highly prevalent CC1-MRSA-IV clone may have emerged locally due to marked similarities between the microarray profiles of CC1-MRSA-IV and CC1-MSSA isolates recovered in the same region (Monecke et al., 2014). Significantly, the Romanian isolates investigated in the present study were also included in the 2014 study. The results of the present study strongly support the conclusions of the 2014 Romanian study. Firstly, the existence of a PVL-negative CC1-MRSA-IV clone in Romania that is distinct from WA MRSA-1/45/57 was confirmed and secondly, the close relatedness of the Romanian CC1-MRSA-IV and Romanian CC1 MSSA (which differed by as few as 47 cgSNPs) was verified (Fig. 1). The available evidence suggests it is likely that this novel CC1-MRSA-IV clone originated in Romania based on the following facts: (i) CC1 MSSA are common in Iaşi, Romania (Monecke et al., 2014), (ii) the CC1-MRSA-IV clone was predominant in Iaşi by 2008 (Monecke et al., 2014), at which time it had been detected in only two patients in Ireland and (iii) none of the Irish CC1-MSSA isolates investigated grouped in close proximity to clade A. However, the lack of published studies on MRSA and/or MSSA from neighbouring countries means the possibility of this clone having originated elsewhere in South-Eastern Europe cannot currently be ruled out.

Following its emergence in South-Eastern Europe, putatively in Romania, the European CC1-MRSA-IV clone spread to Ireland (Fig. 1; Table 1). While this clone was present in the Irish community by 2007, it was not detected in the Irish healthcare system until 2012 and did not become prevalent in Ireland until 2013 (Table S1). These findings support the previously outlined suggestion that consideration of risk-factors relating to the acquisition of MRSA in the community (and not only in healthcare settings e.g. previous hospitalisation, non-intact skin) may be appropriate during targeted MRSA screening in Irish hospitals (Earls et al., 2017; Irish Department of Health, 2013). Interestingly, the Irish clade A isolates (which were recovered between 2007 and 2017) exhibited a relatively high level of genotypic diversity (1–102 cgSNPs), suggesting that the European CC1-MRSA-IV clone may have been introduced into Ireland on several occasions. Indeed, the first two European CC1-MRSA-IV isolates identified in Ireland were recovered just 26 days apart (Table S1) and differed by 52 cgSNPs. Correspondingly, the rate of migration from Romania to Ireland rose dramatically in 2007 and was consistently high until 2017 (Irish National Central Statistics Office, 2009, 2017). Phylogenetic analysis also indicated that the European CC1-MRSA-IV clone spread to Germany (Fig. 1; Table 1). Although only sporadically encountered in Dresden, this clone became prevalent in Regensburg between 2016 and 2018, as demonstrated by its regular detection in the community and in two different hospitals. Remarkably, considering their recovery during a period of approximately two years, the German clade A isolates exhibited a particularly high level of genotypic diversity (12–115 cgSNPs), indicating that the European CC1-MRSA-IV clone may have been introduced into Germany on multiple occasions. Indeed, a 2014 German study demonstrated that 7/51 (14%) Romanian healthcare workers recently employed in a hospital in Aachen were colonised with an unusual MRSA-t127 strain (Scheithauer et al., 2014). In 2015, Romanians represented the second largest group (213,000 people) of foreign nationals living in Germany (Federal Office for Migration and Refugees, Germany, 2016), with a particularly large community living in the Regensburg region.

As previously described, a largely mupirocin-resistant variant of the European CC1-MRSA-IV clone (subclade A1) was responsible for a protracted outbreak in an Irish hospital between 2013 and 2016 (Earls et al., 2017). Isolates of this variant were also recovered from several other hospitals and the community (Earls et al., 2017). As part of the present study, cgSNP analysis revealed that the outbreak continued into 2017, and spread to three additional hospitals (Fig. 1; Table 1). Importantly, this study also revealed that *ileS2*-encoding plasmid (p140355) previously identified among the outbreak isolates also encodes *qacA* and therefore constitutes the first reported plasmid in *S. aureus* to encode resistance to the two antimicrobial agents (i.e. mupirocin and chlorhexidine) commonly used for *S. aureus* decolonisation (Poovelikunnel et al., 2015). While successful read alignment of the 80 *ileS2* and *qacA*-encoding outbreak isolates against the p140355 sequence suggested that all such isolates harboured this plasmid, two outbreak isolates appeared to harbour a variant of this plasmid which did not encode *qacA*. Notably, however, this *qacA*-negative plasmid did not disseminate with the same success as p140355. Therefore, despite the general lack of emphasis on antiseptic-resistance in the literature and public domain, it is possible that the combination of mupirocin and chlorhexidine resistance may have driven the selection of p140355-harbouring MRSA in Ireland.

It is highly likely that the CC1-MRSA-IV clone defined in this study is present in other European countries in addition to Romania, Ireland and Germany. Indeed, PVL-negative ST1-MRSA-IV-t127 have been recovered from humans, cattle, pigs, cow's milk and goat's milk in Italy, and from rooks in Austria (Alba et al., 2015; Basanisi et al., 2017; Cortimiglia et al., 2015; Loncaric et al., 2013; Monaco et al., 2013; Normanno et al., 2015). Furthermore, where detailed, the genotypic characteristics of these PVL-negative ST1-MRSA-IV-t127 correspond to those associated with the European CC1-MRSA-IV clone defined in this study. For example, between 2009 and 2011, PVL-negative ST1-MRSA-IV-t127 which generally harboured *aphA3*, *blaZ*, *sat* and *tet*(K), while exhibiting variable *erm*(C), *sak* and *scn* carriage, were isolated from dairy cows, humans and pigs in Italy (Alba et al., 2015). Indeed, 19% of the non-outbreak (i.e. non-subclade A1) European CC1-MRSA-IV isolates identified during this study did not harbour any human immune evasion genes (i.e. *chp*, *sak* or *scn*), indicating that this clone may be also be prevalent in animals (Table 2). Furthermore, while this study was under review, an Italian study (which included one of the Irish clade A1 isolates investigated in the present study) confirmed the presence of the European CC1-MRSA-IV clone in a paediatric hospital in Florence (Manara et al., 2018). Moreover, in line with the findings of the present investigation, this Italian study estimated that the novel CC1-MRSA-IV clone diverged 6–28 years ago.

The present study has demonstrated the existence of a European PVL-negative CC1-MRSA-IV clone that is distinctly different from the previously characterised and well-defined PVL-negative CC1-MRSA-IV clone, WA MRSA-1/45/57. Furthermore, cgSNP analysis revealed that this MDR clone may have originated in South-Eastern Europe, before spreading to both Ireland and Germany. Finally, the *ileS2*-encoding plasmid which facilitated the spread of this clone in Ireland was determined to also encode *qacA*, highlighting the increasing importance of effective mupirocin and chlorhexidine alternatives for *S. aureus* decolonisation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.021.

Declaration of interest

SM, RE and PS are employees of Abbott (Alere Technologies GmbH). The other authors declare no conflicts of interest.

Author contributions

MRE conceived the study, performed the WGS data analysis and drafted the manuscript. ACS conceived the study and assisted with WGS data analysis and writing the manuscript. DCC conceived the study, purchased the required materials, assisted with data analysis and drafted the manuscript. GB conceived the study assisted with data analysis and drafted the manuscript. AS and WS-B conceived the study, assisted with data analysis and drafted the manuscript. TS and OD conceived the study assisted with data analysis and drafted the manuscript. PS, RE and SM conceived the study, assisted with bioinformatics analysis, microarray analysis data analysis and drafted the manuscript. All authors read and approved the final manuscript.

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Appendix 2

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HEALTHCARE WORKER INFORMATION LEAFLET

Study title: An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

Principal investigator's title name & title: Professor Hilary Humphreys, Beaumont Hospital and the RCSI

Telephone number of principal investigator: 01-xxxxxxx

Co-investigator's tite & name: Professor David Coleman & Dr. Anna Shore, Dublin Dental University Hospital and Trinity College Dublin

- You are being invited to take part in a research study to be carried out at Beaumont Hospital. Before you decide whether or not you wish to take part, you should take sufficient time to read the information below carefully and, if you wish, discuss it with your family, friends or doctor. Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision.
- You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.
- You don't have to take part in this study. If you decide not to take part you will not be penalised in any way and will not give up any benefits that you had before entering the study.
- You can change your mind about taking part in the study at any time. Even if the study has started, you can still opt out. You don't have to give us a reason. If you do opt out, you will not be penalised.

Why is this study being done?

- *Staphylococcus aureus* is a germ, also known as MRSA or MSSA, that is carried by many people in the nose and throat/mouth, without causing them harm. However, it can also cause different types of infections which can be minor or very serious.
- Carriage of this germ is often higher among people working in hospitals. These healthcare workers often have close contact with patients and MRSA/MSSA from their nose and throat/mouth can be transferred to patients.
- This research study is taking place to find out how commonly and for how long MRSA/MSSA is carried in the nose and throat/mouth of healthcare workers and whether some of these are transferred to patients.

Who is organising and funding this study?

- This study is being carried out by researchers at Beaumont Hospital/RCSI and Trinity College Dublin and is funded by a Health Research Board (HRB) grant.
- No pharmaceutical companies are funding this study and we are not being paid to recruit patients.
- This research project has been approved by the Beaumont Hospital Research Ethics Committee.

Why am I being asked to take part?

- You are being asked to take part because you are a healthcare worker who is working on one of the wards in Beaumont Hospital where the study is taking place.
- You will not be asked to take part in the study if you are on antibiotics or have been on antibiotics during the previous month or if there is an outbreak of MRSA/MSSA on the hospital ward where you are currently mainly working on.

How will the study be carried out?

- All samples will be taken by a Research Nurse during a six-week period on each hospital ward. The samples will be taken by gently rubbing a cotton bud on the inside of your nose and using a liquid which you will be asked to use to rinse out your mouth with for 30 seconds and then return to the original container. These procedures will be painless and will take less than one minute.
- You will then be asked to fill in a short questionnaire asking about your age, country of origin, general health issues including any long-term medical conditions, any history of hospitaliation and other healthcare worker related issues. This information will remain confidential.
- The samples will be brought the The Dublin Dental University Hospital Microbiology Laboratory at Trinity College Dublin and will be analysed for the MRSA/MSSA germ by the Research Team.
- Any MRSA/MSSA isolates that we identify from your sample will be stored in a freezer and the cotton buds and rinse solutions will then be destroyed and will not be used for any other purpose.
- Anyone taking part in the study will be assigned a unique number which will be used to label the questionnaire and your samples. You will be sampled a total of four times i.e. every three months for one year. Each time the samples will be taken by the same Research Nurse using the same procedure but you will only need to complete the questionnaire the first time. Only your unique number will be recorded during these follow up screening sessions and not your name. The samples will be processed in the same way each time.
- We will also be seeking nose and mouth samples from patients.

What will happen to me if I agree to take part?

- If you decide to take part you will be asked to sign a consent form. You will then be given a unique code which will be used to label your samples and the questionnaire.
- Your nose and throat/mouth will be sampled by a Research Nurse four times over the next 12 months. Each sampling session will be performed during your normal working day at a time arranged with you and will take less than one minute. You will only be asked to fill in a short questionnaire the first time that the samples are taken and this will take less than 4 minutes.

What are the benefits?

- There are no direct benefits to you if you take part in this study and you will not be paid for your participation. The results of the study will not be made available to you and no treatment will be offered. Your participation in this study will provide research material for a study that will help to improve our understanding of the threat posed by the spread of these germs in hospitals.
- The results of this study will be discussed with Beaumont Hospital Infection Prevention and Control Team and with the Health Service Executive (HSE) and will be used to help in the prevention and control of the spread of this germ in hospitals and to reduce infections in patients.

What are the risks?

- There are no risks associated with sampling the nose using a cotton bud or rinsing the throat/mouth with this rinse liquid.
- The entire procedure, including sampling and completing the questionnaire, will take less than 5 minutes.

Is the study confidential?

- Yes, the study is confidential. Your identity will remain confidential. Your name will only be listed on the consent form. Your name will not be published and it will not be disclosed to anyone.
- Samples will be labelled with a number and the only details that will be recorded will be your age, country of origin and general health and work-related issues. These details will be stored in a password protected computer file and will not be linked to your name. Only the immediate research team will have access to the data. Consent forms and questionnaires will be shredded 1 year after the completion of the project.
- The samples from your nose and mouth will be destroyed once they have been tested for the germ. No genetic testing of your samples will be carried out. Only the germs identified will be kept for future research purposes.
- You will not receive any results from the study but they may be published and presented at scientific meetings. It will not be possible to identify you from any of the results or publications.

Where can I get further information?

- If you have any further questions about the study or if you want to opt out of the study, you can do so at any time and you will not give up any benefits that you had before entering the study.
- If you need any further information now or at any time in the future, please contact:
  Professor Hilary Humphreys 01-xxxxxxx/xxxxxxxxxx@xxxx.xx
  Aoife Kearney (Research Nurse) 08xxxxxxxx/xxxxx.xxxxxxx@xxxxxx.xxx.xx

Website: www.beaumont.ie

Ospidéal Beaumont

BEAUMONT HOSPITAL
P. O. Box 1297 Beaumont Road Dublin 9
Telephone: 809 3000 / 837 7755 Facsimile: 837 6982

PATIENT INFORMATION LEAFLET

Study title: An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

Principal investigator's title name & title: Professor Hilary Humphreys, Beaumont Hospital and the RCSI

Telephone number of principal investigator: 01-xxxxxxx

Co-investigator's title & name: Professor David Coleman & Dr. Anna Shore, Dublin Dental University Hospital and Trinity College Dublin

- You are being invited to take part in a research study to be carried out at Beaumont Hospital. Before you decide whether or not you wish to take part, you should take sufficient time to read the information below carefully and, if you wish, discuss it with your family, friends or doctor. Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision.
- You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.
- You don't have to take part in this study. If you decide not to take part, you will not be penalised in any way and it will not affect in any way your future medical care.
- You can change your mind about taking part in the study at any time and any data or samples collected will not be used in the study. You don't have to give us a reason. If you do opt out, rest assured you will not be penalised.

Why is this study being done?

- *Staphylococcus aureus* is a germ, also known as MRSA or MSSA that is carried by many people in the nose and throat/mouth, without causing them harm. However, it can also cause many different types of infections that can be minor or serious.
- Carriage of this germ is often higher among people who work in hospitals. These healthcare workers often have close contact with patients and MRSA/MSSA from their nose and throat/mouth can be transferred to patients.
- This research study is taking place to find out how commonly and for how long MRSA/MSSA is carried in the nose and throat/mouth of healthcare workers and whether some of these are transferred to patients.

Who is organising and funding this study?

- This study is being carried out by researchers at Beaumont Hospital/RCSI and Trinity College Dublin and is funded by a Health Research Board (HRB) grant.
- No pharmaceutical companies are funding this study and we are not being paid to recruit patients.
- The Beaumont Hospital Research Ethics Committee has approved this research project.

Why am I being asked to take part?

- You are being asked to take part because you are a patient on one of the wards in Beaumont Hospital where the study is taking place.

How will the study be carried out?

- A Research Nurse will take all samples. The samples will be taken by gently rubbing a cotton bud on the inside of your nose and using a liquid which you will be asked to use to rinse out your mouth with for 30 seconds and then return to the original container. These procedures will be painless and will take less than one minute.
- You will then be asked to fill in a short questionnaire asking about your age, country of origin and general health issues including any long-term medical conditions and history of hospitalisation. This information will remain confidential.
- The samples will be brought to the Dublin Dental University Hospital (DDUH) Microbiology Laboratory at Trinity College Dublin and will be analysed for the MRSA/MSSA germ by the Research Team.
- Any MRSA/MSSA germs that we identify from your sample will be stored in a freezer and the cotton buds and rinse solutions will then be destroyed and will not be used for any other purpose.
- If any MRSA/ MSSA germs have been stored in Beaumont Hospital laboratory from samples sent when you previously had an infection or if you currently have an infection, they will also be obtained to be include in this study and they will also be stored in a freezer at the DDUH.
- Everyone taking part in the study will be assigned a unique code that will be used to label his or her questionnaire and samples. On the day of sampling, your name will be used to link the samples we obtain from you with the germs stored when you previously had an infection. Once these germs are obtained from the hospital laboratory, your name will only be retained on the signed consent form.
- We will also be seeking nose and mouth samples from healthcare workers.

What will happen to me if I agree to take part?

- If you decide to take part your will be asked to sign a consent form. You will then be given a unique code that will be used to label your samples and the questionnaire.
- A Research Nurse will sample your nose and throat/mouth at a time arranged with you. This will take less than one minute. You will be asked to fill in a short questionnaire and this will take less than 4 minutes.

What are the benefits?

- There are no direct benefits to you if you take part in this study and you will not be paid for your participation. The results of the study will not be made available to you and no treatment will be offered. Your participation in this study will provide research material for a study that will help to improve our understanding of the threat posed by the spread of these germs in hospitals.
- The overall results of this study will be discussed with Beaumont Hospital Infection Prevention and Control Team and with the Health Service Executive (HSE) and will be used to help in the prevention and control of the spread of this germ in hospitals and to reduce infections in patients.

What are the risks?

- There are no risks associated with sampling the nose using a cotton bud or rinsing the throat/mouth with this rinse liquid.
- The entire procedure, including sampling and completing the questionnaire, will take less than 5 minutes.

Is the study confidential?

- Yes, the study is confidential. Your identity will remain confidential. Your name will be listed on the consent form and will be linked to your nose and mouth samples until the end of the day of sampling so that your clinical isolates can be obtained. Your name will then be replaced with your unique number and it will not be possible to link your samples to you. Your name will not be published in any scientific publications or other documents and it will not be disclosed to anyone.
- The only details that will be recorded will be your age, country of origin, history of hospitalisation and general health issues. These details will be stored in a password protected computer file and will not be linked to your name. Only the immediate research team will have access to the data. Consent forms and questionnaires will be shredded 1 year after the completion of the project.
- Your nose and mouth samples will be destroyed once they have been tested for the germ. No genetic testing of your samples will be carried out. Only the germs identified will be kept for future research purposes.
- You will not receive any results from the study but they may be published and presented at scientific meetings. It will not be possible to identify you from any of the results or publications.

Where can I get further information?

- If you have any further questions about the study or do not wish to participate you can rest assured that your routine care will not be affected in any way.
- If you need any further information now or at any time in the future, please contact:
  Professor Hilary Humphreys 01-xxxxxxx/xxxxxxxxxx@xxxx.xx
  Aoife Kearney (Research Nurse) 08xxxxxxxx/xxxxx.xxxxxxx@xxxxxx.xxx.xx

Appendix 3

Website: www.beaumont.ie

Ospidéal Beaumont

BEAUMONT HOSPITAL
P. O. Box 1297 Beaumont Road Dublin 9
Telephone: 809 3000 / 837 7755 Facsimile: 837 6982

Healthcare Worker Consent Form

Study title: An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study and that I can opt out at any time. I understand that I don't have to give a reason for opting out and I understand that by opting out I won't be penalised.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
I am aware of the potential risks of this research study.	**Yes** ☐	**No** ☐

Storage and future use of information: I give my permission for information collected about me to be securely stored or electronically processed for the purpose of scientific research and to be used in <u>related studies or other studies in the future</u> but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐

Healthcare Worker Name (Block Capitals)	Signature	Date

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above participant the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

Name (Block Capitals)	Qualifications	Signature	Date

2 copies to be made: 1 for participant, 1 for PI.

Website: www.beaumont.ie

Ospidéal Beaumont

BEAUMONT HOSPITAL
P. O. Box 1297 Beaumont Road Dublin 9
Telephone: 809 3000 / 837 7755 Facsimile: 837 6982

Patient Consent Form

Study title: An investigation of the role of *Staphylococcus aureus* colonisation of healthcare workers in the transmission of *S. aureus* to patients using whole-genome sequencing

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study and I understand that not taking part will not affect won't affect my future medical care.	**Yes** ☐	**No** ☐
I am aware of the potential risks of this research study.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
Storage and future use of information: I give my permission for information collected about me to be securely stored or electronically processed for the purpose of scientific research and to be used in <u>related studies or other studies in the future</u> but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐

Patient Name (Block Capitals)	Signature	Date

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

Name (Block Capitals)	Qualifications	Signature	Date

3 copies to be made: 1 for patient, 1 for PI and 1 for hospital records.