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**Comparative Molecular Characterisation of Community
and Healthcare-Associated Panton-Valentine
Leukocidin-Positive *Staphylococcus aureus* and *S.*
aureus from Bloodstream Infections in Ireland**

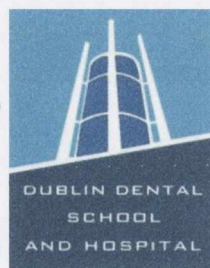
A thesis submitted to the University of Dublin, Trinity College, in fulfilment of the
requirements for the degree Doctor of Philosophy (Ph.D.) by

Sarah Tecklenborg

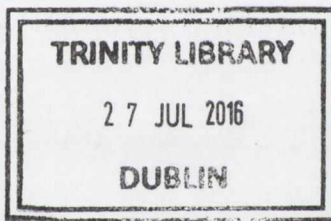
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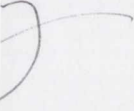


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Abbreviations

ACME	Arginine catabolic mobile element
AR	Antibiogram resistogram
attL/R	Attachment site left/right
BHI	Brain-Heart infusion
BSI	Bloodstream infection
CA	Community associated
CBA	Columbia blood agar
CC	Clonal Complex
Ccr	Cassette chromosome recombinase
CDC	Center for Disease Control and Prevention
cm	Centimeter
CO	Community onset
DNA	Deoxyribonucleic acid
EARS-Net	European antimicrobial resistance surveillance network
<i>et al.</i> ,	and others
etc	et cetera
Fig.	Figure
HCA	Healthcare associated
HO	Hospital onset
I	Intermediate
ICA	Intercellular adhesion cluster
IEC	Immune evasion cluster
kb	Kilobase pair

LA	Livestock associated
MIC	Minimum inhibitory concentration
min	Minute
MGE	Mobile genetic element
ml	Millilitre
MLST	Multi-locus sequence type
MRSA	Methicillin-resistant <i>S. aureus</i>
MSSA	Methicillin-sensitive <i>S. aureus</i>
MSCRAMMs	Microbial surface components recognising adhesive matrix molecules
ng	Nanogram
NICU	Neonatal intensive care unit
nm	Nanometer
NMRSARL	National MRSA reference laboratory
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PSM	Phenol soluble modulin
PVL	Panton Valentine Leukocidin
rpm	Rotations per minute
RNA	Ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SCC	Staphylococcal cassette chromosome
SNP	Single nucleotide polymorphism

SNV	Single nucleotide variation
SSTI	Skin and soft tissue infection
ST	Sequence type
TSS	Toxic shock syndrome
TST	Toxic shock toxin
µg	Microgram
µl	Microlitre
UK	United Kingdom
USA	United States of America
WGS	Whole-genome sequencing

Publications

Some of the original work presented in this thesis has been published in a refereed international publication as listed below. Copies of the manuscripts are included at the end of the thesis.

Brennan GI, Shore AC, Corcoran S, **Tecklenborg S**, Coleman DC, O'Connell B. 2012. Emergence of hospital- and community-associated panton-valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* genotype ST772-MRSA-V in Ireland and detailed investigation of an ST772-MRSA-V cluster in a neonatal intensive care unit. *Journal of Clinical Microbiology* 50:841-847.

Shore AC, **Tecklenborg SC**, Brennan GI, Ehricht R, Moneke S, Coleman DC. 2014. Panton-Valentine leucocidin-positive *Staphylococcus aureus* in Ireland from 2002 to 2011: 21 clones, frequent importation of clones, temporal shifts of predominant methicillin-resistant *S. aureus* clones , and increasing multiresistance. *Journal of Clinical Microbiology*. 52(3):859-870.

Chapter 1

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is one of the most frequently isolated bacterial pathogens from healthcare- and community-associated infections (HCA and CA, respectively) worldwide and is a serious burden for patients and the healthcare system. *Staphylococcus aureus* is a coagulase-positive staphylococcal species, producing the protein coagulase, a prothrombin activator that converts fibrinogen to fibrin resulting in clotting of blood plasma. Its main ecological niche in humans is the moist squamous epithelium of the anterior nares where it colonises approximately 30% of the population (Mainous *et al.*, 2006). *Staphylococcus aureus* also forms part of the normal flora of the throat, gastrointestinal tract, axilla, perineum and groin. Not only does *S. aureus* live commensally with its host but it is also considered to be the most pathogenic of the staphylococci and given the chance to permeate the skin's natural barriers it is capable of causing a wide variety of infections. These range from superficial skin and soft tissue infections (SSTIs) to the more serious and life-threatening conditions such as toxic shock, endocarditis, osteomyelitis, septicaemia and necrotising pneumonia. Transmission of *S. aureus* commonly occurs through direct contact from person to person, through interaction with contaminated surfaces and fomites and through contact with livestock and companion animals (Lowy, 1998; Uhleman *et al.*, 2011).

1.2 Methicillin-resistant *S. aureus*

In the 1940's *S. aureus* isolates exhibiting resistance to penicillin, the first discovered β -lactam antibiotic, were identified and this resistance was subsequently found to be due to the production of a penicillinase enzyme (Kirby, 1944; Barber & Rozwardowska-Dowzenko, 1948). This led to the development of antibiotics such as the semi-synthetic penicillinase-resistant beta-lactams methicillin and oxacillin and the first and second generation cephalosporins. However, as with penicillin, resistant *S. aureus* strains emerged very shortly after the introduction of these antibiotics into clinical practice. The first methicillin-resistant *S. aureus* (MRSA) isolates were detected in the United Kingdom (UK) in 1961 just two years after methicillin was introduced into clinical practice (Jevons, 1961). Subsequently different MRSA clones emerged, which exhibited resistance to a range of antimicrobial agents and many different strains spread worldwide and have become endemic in many regions. Today, nosocomial outbreaks of sometimes

multidrug-resistant MRSA are common. Prevalence levels of MRSA among *S. aureus* infections vary in different countries ranging from <1% in Denmark (Faria *et al.*, 2005) to >70% in Korea (Chen & Huang, 2014). In Europe in 2007 there were 27,711 cases of MRSA bacteraemia, which were responsible for 5,503 deaths and 255,683 extra hospital days (Marliecke *et al.*, 2011). The proportion of MRSA among *S. aureus* bloodstream infections (BSIs) across Europe ranges from less than 1% in Norway to greater than 50% in Romania (Fig. 1.1(A)). Currently in Ireland 20.3% of *S. aureus* BSIs in hospitals are caused by MRSA (Fig. 1.1(B)).

1.2.1 The mechanism of methicillin resistance in *S. aureus*

Methicillin resistance in *S. aureus* is due to the presence of the *mec* gene which encodes a modified penicillin binding protein termed PBP2' or PBP2a. Penicillin binding proteins catalyse the synthesis and crosslinking of peptidoglycan in the bacterial cell wall via transglycosylation and transpeptidation, respectively (Hartman & Toomas 1984; Scheffers & Pinho 2005; Reed *et al.*, 2015). The extra PBP in MRSA (PBP2a) is a variant of PBP2, the only PBP with both transglycosylase and tranpeptidase functions (Hartman & Tomasz, 1984). PBP2a has a very low affinity for beta-lactams and while the four native *S. aureus* PBPs are inactivated by methicillin, the production of PBP2a by MRSA permits cell wall synthesis to continue (Hartman & Tomasz, 1984). Production of PBP2a confers resistance to all β -lactam antibiotics except ceftobiprole (El Solh, 2009).

Three *mec* gene types have been described to date, *mecA*, *mecB* and *mecC*, each sharing 60-70% DNA sequence identity (Ito *et al.*, 2012). Within each *mec* gene type a number of allotypes have been identified which share between 70% and 95% DNA sequence identity to *mecA* of *S. aureus* N315, *mecB* of *Micrococcus caseolyticus* JCSC5402 and *mecC* of *S. aureus* LGA251 (Ito *et al.*, 2012). Both *mecA* and *mecC* have been found in *S. aureus* and coagulase-negative staphylococci (CoNS), while *mecB* has been identified in *Micrococcus caseolyticus* only (Baba *et al.*, 2009; Ito *et al.*, 2012). The *mecA* gene is the most prevalent *mec* gene type in *S. aureus* and is present in the majority of MRSA and methicillin-resistant CoNS, while *mecC* was only described recently, predominantly in association with *S. aureus* lineages from animals (Garcia-Alvarez *et al.*, 2011; Shore *et al.*, 2011; Patterson *et al.*, 2014; Espinosa-Gongora *et al.*, 2015).

In *S. aureus*, the *mec* gene forms part of the *mec* gene complex and is located on a mobile genetic element (MGE) known as the Staphylococcal Cassette Chromosome *mec*

(SCC*mec*) that integrates precisely into an open reading frame (orf) within the staphylococcal genome designated orfX. Each SCC*mec* element comprises (i) a *mec* gene complex consisting of a *mec* gene, in most instances the *mec* regulatory genes *mecI*, *mecR1* and *mecR2* and insertion sequences IS431 or IS1272 and (ii) a cassette chromosome recombinase (*ccr*) gene complex responsible for the mobility and precise integration and excision of the SCC*mec* cassette (IWG-SCC, 2009). Four *mec* gene complexes (designated class A, B, C and E *mec*) and seven *ccr* complexes (designated *ccrA1B1*, *ccrA2B2*, *ccrA3B3*, *ccrA4B4*, *ccrA1B6*, *ccrA1B3* and *ccrC*) have been described to date in *S. aureus* (<http://www.sccmec.org/>). Each SCC*mec* type has a unique combination of *ccr* and *mec* complex genes and 11 different SCC*mec* types (SCC*mec* types I-XI) have been characterised to date in MRSA (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004; Boyle-Vavra *et al.*, 2005; Shore *et al.*, 2005; Oliveira *et al.*, 2006; Berglund *et al.*, 2008; Zhang *et al.*, 2009; Shore *et al.*, 2011). The *mecA* gene has been associated with SCC*mec* types I-X and *mecC* with SCC*mec* XI. Numerous SCC*mec* subtypes have also been identified and these differ in the joining (J) regions and can contain integrated plasmids and transposons harbouring additional antimicrobial resistance determinants (IWG-SCC, 2009).

1.3 Evolution and epidemiology of *S. aureus*

Methicillin-resistant *S. aureus* have emerged from methicillin-susceptible *S. aureus* (MSSA) on multiple independent occasions via acquisition of SCC*mec* (Enright *et al.*, 2002; Robinson & Enright 2003). Evidence to date indicates that CoNS are a reservoir for SCC*mec* in *S. aureus* (Tsubakishita *et al.*, 2010). While MRSA was traditionally considered to be a nosocomial pathogen causing infections in elderly and immunocompromised patients, since the 1990s there has been a significant increase in the numbers of CA-MRSA infections in otherwise healthy individuals outside of the hospital environment (Adcock *et al.*, 1998; CDC, 1999; Frank *et al.*, 1999; Charlebois *et al.*, 2002; Baggett *et al.*, 2003; CDC, 2003; Buckingham *et al.*, 2004; Dietrich *et al.*, 2004; Fridkin *et al.*, 2005). Additionally, MRSA has also emerged among animals, including pets, livestock (livestock-associated (LA) MRSA) and wildlife, with the spread of MRSA between animals and humans being reported with increasing frequency (Aarestrup 2015; Misic *et al.*, 2015; Koyama *et al.*, 2015; van Cleef *et al.*, 2015). There are significant differences between HCA-, CA- and LA MRSA, not only with respect to their patient

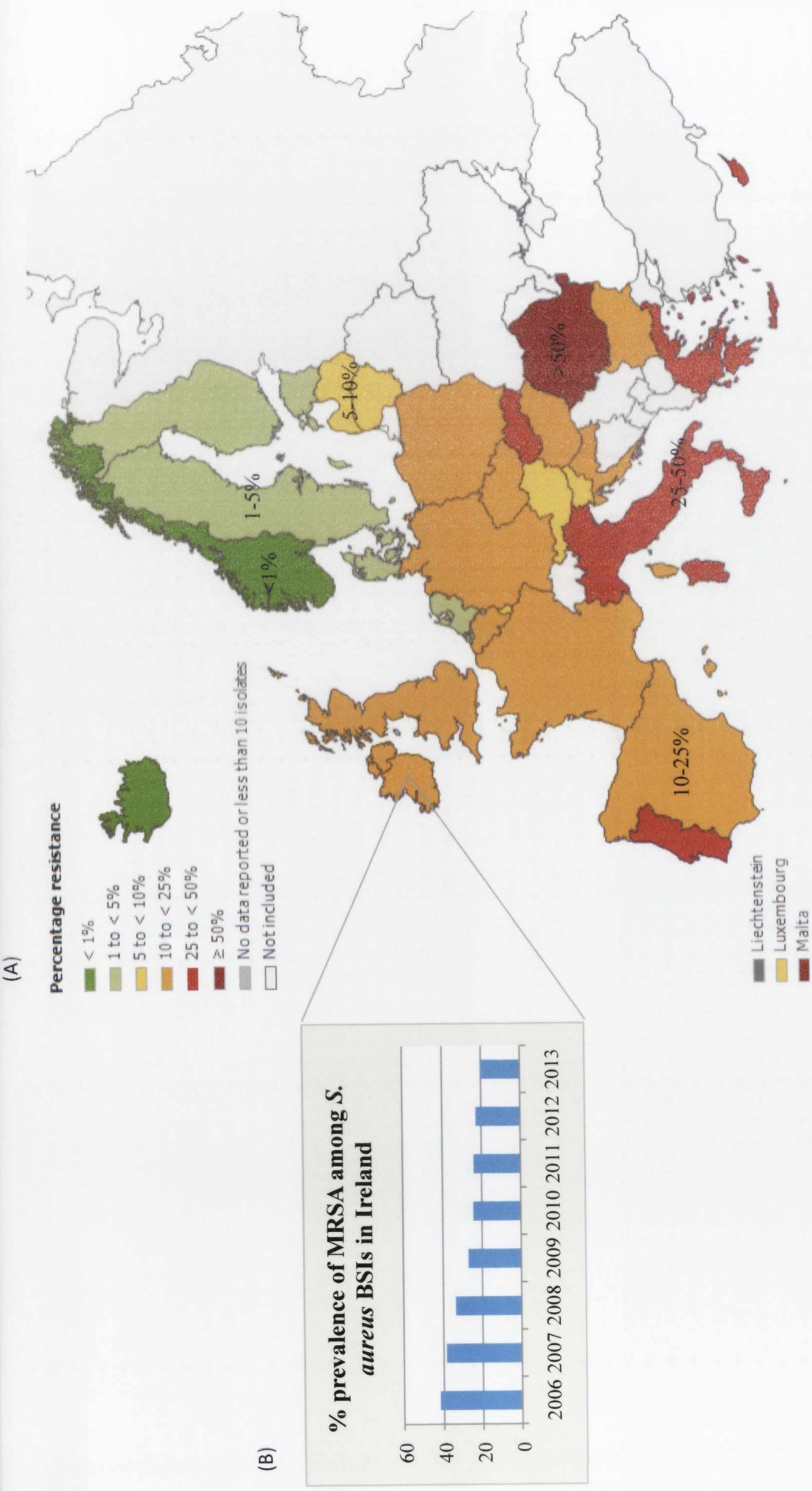


Figure 1.1 Prevalence of MRSA among *S. aureus* bloodstream infections (A) in Europe in 2013 and (B) in Ireland between 2006 and 2013. Adapted from EARS-net (2013) and HPSC (2014).

profiles but also genetically (Table 1.1); HCA and CA-MRSA are described in more detail below due to their relevance to the present study and the key differences are highlighted in Table 1.1.

1.3.1 Healthcare-associated MRSA

Patients with HCA-MRSA infections typically have the traditional risk factors for MRSA infections such as older age, exposure to broad spectrum antibiotics, having an underlying illness, recent surgery or an immunocompromised state or attending a healthcare facility (CDC, 2005; Otter & French, 2010). Healthcare-associated MRSA infections are often attributed to poor infection control and prevention practices (Edgeworth *et al.*, 2007). Improved hand hygiene alone has been shown to reduce the spread of MRSA transmission in certain healthcare facilities (Gagne *et al.*, 2010). Good antimicrobial stewardship also plays a key role in prevention and control of MRSA infections in patients e.g. antibiotic exposure in individual patients in hospitals was found to be associated with 1.8-fold increased risk of subsequent acquisition of MRSA (Tacconelli *et al.*, 2008). The prevalence of MRSA in hospitals has been linked to levels of consumption of specific antibiotics including fluoroquinolones, cephalosporins, amoxicillin and macrolides (MacKenzie *et al.*, 2007; Aldeyab *et al.*, 2012). Programmes targeting antibiotic consumption within hospitals have been associated with a reduction in colonisation and infection rates, with these results most noticeable after a reduction in the use of fluoroquinolones and beta-lactams (Harbarth & Samore, 2008; Gould, 2009).

Certain MRSA clones have been identified as being more specialised and suited to the restricted ecological niches of the hospital environments where survival is dependent on efficient transmission between patients and resistance to the antibiotics associated with a nosocomial environment (Otto, 2013). The majority of HCA-MRSA worldwide belong to just five lineages designated clonal complexes (CCs) 5, 8, 22, 30, 45 (Enright *et al.*, 2002) and within these eight major international HCA-MRSA clones have been identified (Table 1.1). These pandemic HCA-MRSA clones are commonly designated based on their sequence type (ST) as defined by multilocus sequence typing (MLST) (section 1.5.2) and their SCCmec type (Table 1.1). Different HCA-MRSA clones predominate in different geographical regions and some of these MRSA clones have spread worldwide. The factors affecting this regional variation are unknown but may be linked to differences in antimicrobial prescription policies and infection control practices. The emergence of

new HCA-MRSA strains in a particular region is often linked to the importation of new strains (Harris *et al.*, 2012; Köser *et al.*, 2012). Increased and improved transport and worldwide migration have also facilitated the spread of HCA-MRSA clones to new regions.

1.3.1.1 ST22-MRSA-IV

ST22-MRSA-IV is an important pandemic HCA-MRSA clone also known as the UK epidemic strain EMRSA-15. A Portuguese study of infection and screening isolates from patients in 11 hospitals in 2006 found that over 50% of *S. aureus* recovered were ST22-MRSA-IV (Aires-de-Sousa *et al.*, 2008). Similarly in Malta ST22-MRSA-IV accounted for over 50% of a random selection of *S. aureus* isolates recovered from infection and screening specimens in Malta's only tertiary care hospital in 2007/08 (Scicluna *et al.*, 2010). In the UK, ST22-MRSA-IV accounted for 60% of nosocomial BSIs within two decades of its emergence (Johnson *et al.*, 2001) and since 2002 has accounted for over 80% of MRSA from BSIs in patients in Irish hospitals (NMRSARL, 2012). In addition, ST22-MRSA-IV was the most frequently identified MRSA clone in a European study performed in 2006 of 1130 MRSA isolates from patients with BSIs across 25 countries (Grundmann *et al.*, 2010). The ST22-MRSA-IV clone also spread from hospitals into the community and has been recovered on public transport and from companion animals such as dogs and cats (Walther *et al.*, 2008; Wolter *et al.*, 2008). The ST22-MRSA-IV clone's ability to replace other well-established HCA-MRSA clones has been repeatedly shown though the exact reasons for this are still unknown (Hsu *et al.*, 2005; Aires-de-Sousa *et al.*, 2008; Witte *et al.*, 2008). The selective pressures of broad-spectrum antibiotics appear to have driven the successful spread of ST22-MRSA-IV including fluoroquinolone use and subsequent mutations, mutations in the promoter of the macrolide resistance-encoding *erm(C)* gene and a mechanism for oxazilidinone resistance (Holden *et al.*, 2013).

1.3.2 Community-associated *S. aureus*

The success of MRSA clones in the community setting is driven by their ability to disseminate amongst, and infect, a predominantly healthy population. The first reports of CA-MRSA emerged in the 1990s following several outbreaks of infections of MRSA among healthy patients in the community including young children in the United States of America (USA) and Aboriginals in Western Australia (Udo *et al.*, 1993; Adcock *et al.*,

Table 1.1 Epidemiological and genetic differences between healthcare-associated (HCA) and community-associated (CA) MRSA

	HCA-MRSA	CA-MRSA
Clinical manifestations	Pneumonia, urinary tract infections, bloodstream infections, surgical site infections	Skin and soft tissue infections, necrotising pneumonia/fasciitis, sepsis
Risk groups	The elderly, immunocompromised, preterm neonates, post surgical	The young, drug users, prisoners, athletes, soldiers
Risk factors	Indwelling devices, catheters, long-term antibiotic use, haemodialysis, proximity to MRSA positive patient	Close physical contact, abrasion injuries, poor communal hygiene
Antimicrobial resistance	Commonly resistant to multiple antimicrobial agents	Commonly susceptible to multiple antimicrobial agents but multiresistant strains have emerged
SCCmec type	I, II, III & IV	IV, V & VII
PVL toxin	Usually absent	Usually present
Genotypes of major pandemic clones¹ (pseudonym)	ST5-MRSA-II ST8-MRSA-II (Irish AR13/14) ST22-MRSA-IV (Irish AR06/EMRSA-15) ST36-MRSA-II (Irish AR07/EMRSA-16) ST45-MRSA-IV ST239-MRSA-III (Brazilian/Hungarian clone) ST247-MRSA-I (Iberian clone) ST250-MRSA-I (Archaic clone)	ST1-MRSA-IV (USA400/Western Australian Clone) ST8-MRSA-IV (USA300) ST30-MRSA-IV (Southwest Pacific clone) ST80-MRSA-IV (European clone) ST93-MRSA-IV (Queensland clone)

¹Genotypes as defined by multilocus sequence typing, represented by the abbreviation ST (sequence type) and SCCmec typing, indicated with a roman numeral.

1998). These early CA-MRSA clones soon spread worldwide and were followed by the localised emergence of further CA-MRSA clones, many of which also became pandemic.

Patients with CA-MRSA lack the traditional risk factors for HCA-MRSA and infections have been reported among children attending crèches, inmates of jails, families living in close contact and participants of team sports (Kluytmans-Vandenbergh & Kluytmans, 2006). Community-associated MRSA have distinct genetic backgrounds to HCA-MRSA and have emerged from local CA-MSSA rather than the spread of HCA-MRSA into the community. They carry smaller *SCCmec* elements, predominantly *SCCmec* IV or V, that are presumed to be more mobile and infer a fitness advantage to CA-MRSA, although data in support of this are scarce (Berglund & Söderquist, 2008). Community-associated MRSA tend to be resistant to fewer non- β -lactam antibiotics than HCA-MRSA, although this is not useful as a differential marker as some recent CA-MRSA clones exhibit multiresistance, e.g. the Bengal Bay clone ST772-MRSA-V (Prabhakara *et al.*, 2013). Lastly, CA-MRSA often express the virulence factor Panton-Valentine leukocidin (PVL) (section 1.4.3.1).

However, despite these molecular differences the ability to distinguish between HCA- and CA-MRSA using molecular means alone is decreasing. Some HCA-MRSA carry *SCCmec* IV which is often found among CA-MRSA, including the pandemic ST22-MRSA-IV clone, thus *SCCmec* typing is not suitable for distinguishing between CA- and HCA-MRSA. The PVL toxin has been found to be a poor marker for CA-MRSA as not all CA-MRSA harbour the *pvl* locus (Rossney *et al.*, 2007). Complicating this further is the fact that so called HCA-MRSA strains are now causing infections in community settings and CA-MRSA strains are causing infections in hospitals (Schlebusch *et al.*, 2010; Patel *et al.*, 2013). Thus, it is important that the epidemiological characteristics of MRSA infections are analysed together with the molecular characteristics of the isolates in order to distinguish between HCA- and CA-MRSA infections. For example, the Centers for Disease Control and Prevention (CDC) defines CA-MRSA as strains which have been isolated in an outpatient setting or within 48 hours of admission to a hospital. The patient's medical history must show no evidence of MRSA colonisation or infection, previous hospitalisation, dialysis, surgery or residence in a long-term care facility. There must be no risk factors for MRSA such as indwelling devices, long term antibiotic use or catheters (CDC, 2005; Morrison *et al.*, 2006). In addition, HCA- and CA-MRSA can also be distinguished by the clinical manifestations of their infections. HCA-MRSA tend to

cause pneumonia as well as urinary tract, bloodstream and surgical site infections. Up to 90% of CA-MRSA infections are SSTIs (David & Daum, 2010). The more serious conditions of necrotising pneumonia, sepsis and necrotising fasciitis have been reported in association with CA-MRSA (Nastaly *et al.*, 2010).

The success of pandemic CA-MRSA strains has been attributed to a number of factors. These range from the smaller SCCmec elements to carriage of less antibiotic resistance genes, and the expression of specific virulence factors such as the arginine catabolic mobile element (ACME), phenol-soluble modulins (PSM α), the accessory gene regulator (encoded by *agr*), leukocidins A and B (encoded by *lukAB*), protein A (encoded by *spa*), alpha (α) toxin (encoded by *hla*) and in particular PVL (encoded by *lukF/S-PV*) (Diep & Otto, 2008). The epidemiological link between particular CA-MRSA infections such as SSTIs and necrotising pneumonia strongly point to PVL, a potent cytolytic toxin, as playing a significant role in the success of some CA-MRSA and this is discussed in more detail in section 1.4.3.1. Phenol soluble modulins are a group of peptides which recruit and lyse neutrophils during infection (Wang *et al.*, 2007). While HCA-MRSA often do not produce PSMs or produce them at reduced levels, they are expressed at much higher levels in CA-MRSA. Experiments which over expressed α -type PSMs in HCA-MRSA increased the leukocidal activity of these strains relative to those seen in CA-MRSA (Wang *et al.*, 2007). The devastating impact of PSMs in CA-MRSA disease has been demonstrated in bacteraemia and abscess mouse infection models (Wang *et al.*, 2007). Alpha toxin, which promotes host chemokine production by recruiting high levels of neutrophils, has been shown to have a key impact on the outcome of necrotising pneumonia caused by CA-MRSA (Wardenburg *et al.*, 2007; Wardenburg *et al.*, 2008). Both α -toxin and PSM- α are under the control of the global regulator gene *agr* and CA-MRSA strains have been found to strongly express the *agr* molecule RNAIII thus leading to the increased expression of these toxins (Wang *et al.*, 2007).

1.3.2.1 Major CA-MRSA clones

Originally the emergence of CA-MRSA clones appeared to be continent-specific with ST8-MRSA-IV (USA300) and ST1-MRSA-IV (USA400) being associated with the USA, ST80-MRSA-IV with Europe and ST30-MRSA-IV with Oceania (Rasigade *et al.*, 2010). However, these CA-MRSA clones have subsequently spread worldwide (Ellington *et al.*, 2010; Rasigade *et al.*, 2010; Monecke *et al.*, 2011). The ST8-MRSA-IV clone, commonly

referred to as USA300, is the most prevalent and successful pandemic CA-MRSA clone (Moran *et al.*, 2006). In an incredibly short period of time ST8-MRSA-IV replaced other CA-MRSA clones such as ST1-MRSA-IV, which was the most prevalent CA-MRSA clone in the USA up to 2001, and has become the most common cause of all MRSA infection, both HCA and CA, in the USA (Diep *et al.*, 2006; Moran *et al.*, 2006; Popovich *et al.*, 2008). Phylogenetic analysis has indicated that USA300 first diverged from a most common recent ancestor in 1993 (Tewhey *et al.*, 2012; Prosperi *et al.*, 2013). These studies also identified that discrete changes in the core genome of these ST8-MRSA-IV isolates significantly altered the proliferation, persistence and virulence of isolates. The invasiveness of ST8-MRSA-IV was found to be dependent, to some extent, on the genetic distance a particular clone was from the parental USA300 strain. Accumulations of genetic variations were identified in a haplogroup, evolved from the parental strain, which were associated with milder SSTI phenotypes than those isolates which had less genetic variation and caused more severe manifestations (Tewhey *et al.*, 2012). Similar to ST22-MRSA-IV, fluoroquinolone use was identified as a critical driver for the recent evolution and spread of ST8-MRSA-IV (Uhlemann *et al.*, 2014).

It appears that ST8-MRSA-IV added to the burden of infections in hospitals in the USA as opposed to replacing all existing HCA-MRSA strains (Klein *et al.*, 2007) and that this correlated with an increase in the prevalence of MRSA infection in the USA during the last decade. Over 50% of staphylococcal disease in the USA has been found to be caused by MRSA (Klein *et al.*, 2007; Klevens *et al.*, 2007). ST8-MRSA-IV has also become a pandemic CA-MRSA clone with infections being reported in every continent except Antarctica (Pan *et al.*, 2005; Furtado *et al.*, 2006; King *et al.*, 2006; Hota *et al.*, 2007; Liu *et al.*, 2008). The international spread of CA-MRSA clones appears to be due to increased travel and the influx of immigrants and asylum seekers (Dsouza *et al.*, 2010; Ellington *et al.*, 2010).

New multiresistant PVL-positive CA-MRSA clones have also recently emerged including ST772-MRSA-V (the Bengal-Bay Clone) that has spread through countries across several continents in a very short period of time (Dsouza *et al.*, 2010; Ellington *et al.*, 2010). Originally reported in Bangladesh in the community this CA-MRSA clone quickly spread to India causing infections in both hospitals and the community and has, along with *pvl*-positive ST22-MRSA-IV, become the predominant MRSA (Afroz *et al.*, 2008). Since its first identification in 2008, ST772-MRSA-V has been associated with

HCA- and CA-MRSA infections in Germany, Malaysia, Ireland, Italy, Hong Kong, England, Abu Dhabi and Australia and in many cases isolates were recovered from patients with links to India (Ellington *et al.*, 2011; Monecke *et al.*, 2011).

1.3.3 Methicillin-susceptible *S. aureus*

Comparatively little is known about the molecular epidemiology of MSSA clones. In contrast to the clonal clustering seen in MRSA, MSSA isolates show widespread genotypic diversity with no dominant geographical pattern to their spread (Grundmann *et al.*, 2010). Despite this, in some regions, the predominant MSSA strains are frequently analogous to the major MRSA lineages (Miko *et al.*, 2013). The emergence of MRSA from MSSA is a relatively rare event and as such there are fewer MRSA clones compared to MSSA (Enright *et al.*, 2002; Nübel *et al.*, 2008). MSSA are also much older on an evolutionary time scale than MRSA clones, with the majority of MRSA clones being less than 50 years old and, as such, have had less time to diversify (Grundmann *et al.*, 2010). The broader dissemination of MSSA clones within a community could be representative of the younger more mobile patient profile found with MSSA infections (Robotham *et al.*, 2007; Grundmann *et al.*, 2010). A European study of BSIs found significant differences in the patient profile of those with MSSA and MRSA infections. Patients with MSSA were younger and 2.4 times less likely to have their infection associated with hospital acquisition. They also had lower mortality than those with MRSA infection though this could be based on the younger healthier patient profile (Grundmann *et al.*, 2010). In contrast some studies have found MSSA clones to cause more serious infection than MRSA and this has been linked to the fact that MSSA clones commonly harbour and express more virulence genes than MRSA, whereas MRSA tend to sacrifice virulence for increased resistance (Wehrhahn *et al.*, 2012).

1.4. Factors leading to the success of *S. aureus* as a pathogen

The success of *S. aureus* as a pathogen is a complicated process involving the emergence and rapid dissemination of new and often fitter clones as well as an ability to adapt and respond to the changing environment. The latter is due to the extensive range of virulence factors that *S. aureus* can express as well as its ability to develop resistance to antimicrobial agents, many very soon after they are introduced into clinical practice (DeLeo & Chambers, 2009). Many *S. aureus* resistance and virulence genes are carried

on mobile genetic elements (MGEs) that can be horizontally transferred between *S. aureus* strains and from other staphylococcal species (Table 1.2). Much of the genetic variation between *S. aureus* strains is due to the presence of various MGEs such as bacteriophage, SCC/SCC*mec* elements, pathogenicity islands, transposons, insertion sequences and plasmids (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Lindsay & Holden 2004).

The fitness of a given clone is often environment-specific and what makes one clone particularly suited to a given niche, such as antimicrobial resistance in hospitals or virulence factors enabling it to infect younger healthy patients in the community, may not provide a benefit in another niche. Thus in an ever changing environment, emerging clones with traits which give them an advantage over others are selected for over time.

1.4.1 *S. aureus* fitness

When *S. aureus* isolates are in competition with each other, those that are less fit or less well adapted to a specific environment become replaced by better adapted clone (Albrecht *et al.*, 2011). These clonal waves, where one dominant clone is replaced by another, are a common feature of MRSA epidemiology. Fit MRSA clones emerge from prevalent MSSA clones, undergo rapid clonal expansion and widespread dissemination only to subsequently diminish or be replaced. The factors driving the decline and subsequent replacement of specific clones are multifaceted and poorly understood, but despite this the changing epidemiology of *S. aureus* has been widely reported. In Portugal, four successive clonal replacements have occurred ST239-MRSA-III, which was dominant up to 1992/3, was replaced by ST247-MRSA-Ia. This in turn was replaced following the introduction of ST239-MRSA-III (the Brazillian clone) and most recently ST22-MRSA-IV has become the predominant MRSA in Portugal (Aires-de-Sousa *et al.*, 2004; Aires-de-Sousa *et al.*, 2008; Espadinha *et al.*, 2013). Two clones have predominated in the UK since the 1990s, ST22-MRSA-IV and ST30-MRSA-IV, which together accounted for over 95% of nosocomial infections (Ellington *et al.*, 2010). Both clones started to decline in 2004, with ST30-MRSA-IV declining at a faster rate, and no aspects of hospital infection prevention and control policy could be linked to the combined decline (Ellington *et al.*, 2010; Wyllie *et al.*, 2011).

Factors that could potentially drive clonal replacement include resource competition and ecological niche e.g. ST36-MRSA-II was said to be highly suited to the

hospital environment (Bacon & Beasley 2004). Following the introduction of measures to reduce HCA-MRSA bacteremia in the UK ST36-MRSA-II began to decline but other co-circulating strains such as ST22-MRSA-IV did not decline to a similar extent (Rao *et al.*, 2011).

1.4.2 Antimicrobial agent resistance

Antibiotic resistance in *S. aureus* was first identified soon after the introduction of penicillin into routine hospital treatment protocols for infections in the early 1940s (Kirby, 1944) (Fig. 1.2). Resistance to penicillins was mediated by a plasmid-encoded penicillinase which hydrolysed the beta-lactam ring of penicillin. Penicillin-resistant strains became pandemic by the early 1950s (Roundtree & Freeman, 1956). Following the introduction of methicillin as a treatment for penicillin-resistant *S. aureus* infections, methicillin resistance was identified among *S. aureus* in 1961 (Jevons, 1961). The increased pressure caused by MRSA infections in hospitals led to the development of the antibiotic vancomycin in 1954 and in 1997 MRSA emerged which exhibited some resistance to vancomycin (Hiramatsu *et al.*, 1997). Almost as soon as antimicrobials are developed and introduced into clinical practice for *S. aureus* infections, resistance emerges. Antimicrobial resistance is facilitated through the acquisition of resistance genes and/or the development of spontaneous mutations which can modify or protect drug targets, directly modify antibiotics or facilitate the removal of the antibiotic from the bacterial cell (Blair *et al.*, 2015). *Staphylococcus aureus* isolates can harbour a wide range of antimicrobial resistance genes, often carried on mobile genetic elements (Table 1.2) (section 1.4) facilitating the horizontal transfer of such genes between isolates.

Whilst antimicrobial resistance in *S. aureus* provides it with a unique advantage in the hospital environment, the development of resistance through the acquisition of mutations or resistance genes often results in a reduced fitness and a decrease in growth rate (Anderson & Levin., 1999). Ender *et al.* (2004) demonstrated the reduced growth rate which resulted from the gain of SCCmec by a *S. aureus* strain. Different SCCmec elements infer different fitness costs. SCCmec I is associated with a lower growth rate and increased glucose consumption in comparison to strains carrying SCCmec-IV elements (Ender *et al.*, 2004). Kusuma *et al.* (2007) highlighted the fitness costs of two different mutations in *femA* genes which led to lysostaphin resistance in two *S. aureus* variants. Here the fitness cost was highlighted not only through a decline in growth rate

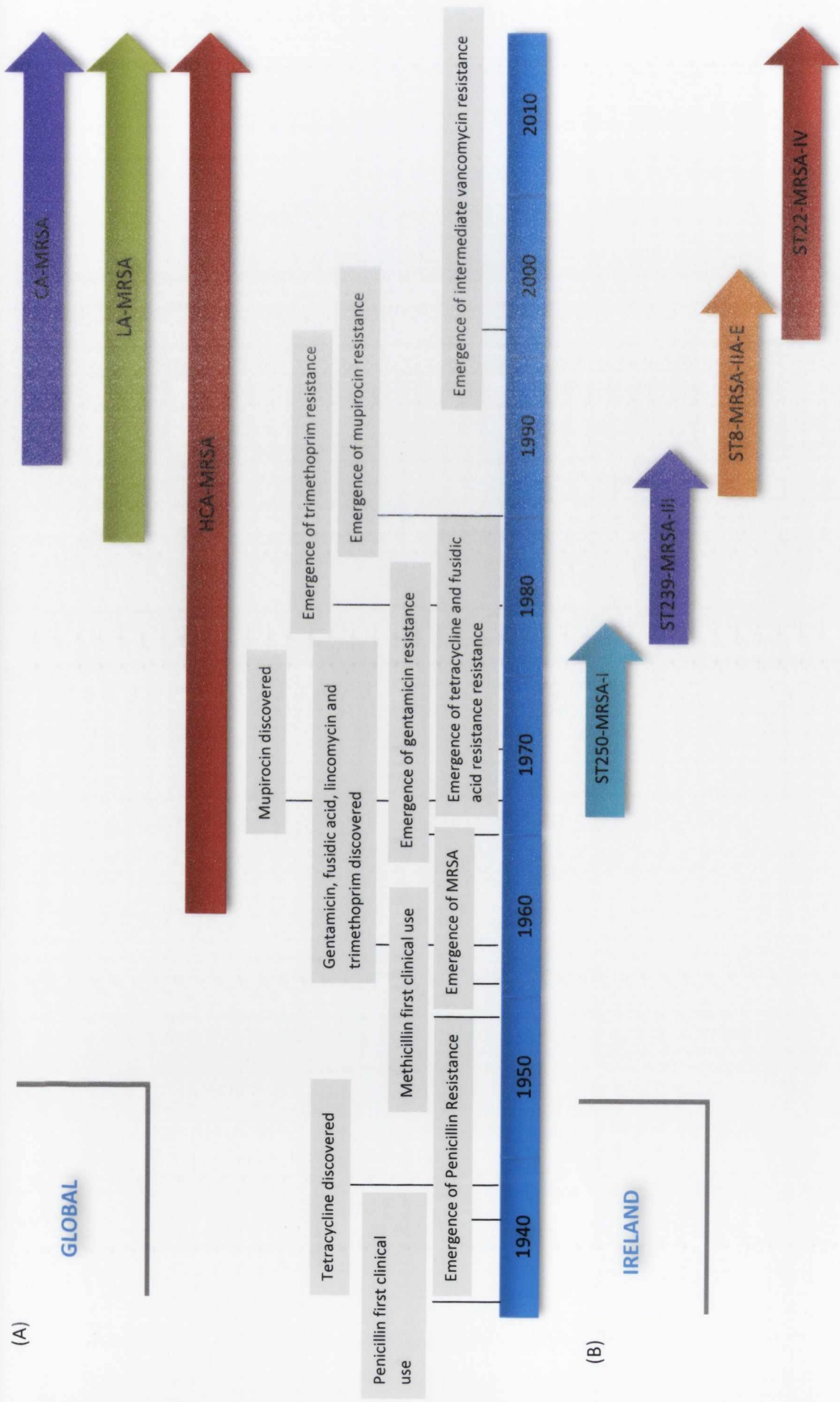


Figure 1.2 Timeline showing (A) the global emergence of healthcare-associated (HCA), community-associated (CA) and livestock-associated (LA) MRSA and the evolution of antibiotic resistance and (B) the major clonal lineages of MRSA that predominated in Irish hospitals since 1971. Adapted from DeLeo & Chambers 2009, Shore *et al.*, 2005 and NMRSARL 2014.

Table 1.2 Examples of *Staphylococcus aureus* antimicrobial resistance and virulence-associated genes encoded on mobile genetic elements¹

Function	Gene	Virulence/Antimicrobial resistance gene product/function	Associated mobile genetic element
Antimicrobial resistance genes	<i>blaZ</i>	β-lactamase	Plasmid
	<i>erm</i> (A),(B),(C)	Resistance to MLS compounds	Plasmid
	<i>lnu</i> (A)	Lincosamide resistance	Plasmid
	<i>msr</i> (A)	Methionine sulfoxide reductase resistance	Plasmid
	<i>mph</i> (C)	Macrolide phosphotransferase	Plasmid
	<i>aacA-aphD</i>	Resistance to aminoglycosides	Transposon/plasmid
	<i>aadD</i>	Resistance to aminoglycosides	Plasmid
	<i>dfrS1</i>	Trimethoprim resistance	Plasmid
	<i>mupA</i>	High-level mupirocin resistance	Plasmid
	<i>tet</i> (K)	Tetracycline resistance	Plasmid
	<i>fosB</i>	Fosfomycin resistance	Plasmid
	<i>qacA,C</i>	Resistance to quaternary ammonium compounds	Plasmid
	<i>cfr</i>	PhLOPSa ² resistance phenotype	Plasmid
	<i>mec</i>	Methicillin resistance	Staphylococcal cassette chromosome
Superantigens	<i>tst</i>	Toxic shock toxin	PI
	<i>entA</i>	Enterotoxin A	Phage
	<i>entB,C,D,E,G,H,I,J,K,L,M,N,O,Q,R,U,Y,X</i>	Enterotoxins	PI & Phage
Immune evasion	<i>sak</i>	Staphylokinase	Phage
	<i>chp</i>	Chemotaxis inhibitory protein	Phage
	<i>scn</i>	Staphylococcal complement inhibitor	Phage
Two-component toxins	<i>lukF-PV</i>	Panton-Valentine leukocidin	Phage
	<i>lukS-PV</i>	Panton-Valentine leukocidin	Phage
	<i>lukM</i>	Leukocidins	Phage
	<i>lukD,E,X,Y</i>	Leukocidins	Genomic island
Miscellaneous virulence factors	<i>etA,B,D</i>	Exfoliative toxins	Phage & PI
	<i>edinA,B,C</i>	Epithelial diffusion inhibitors	PI and plasmids
	<i>arcA,B,C,D</i>	ACME	SCC
	<i>spl</i>	Serine protease	Genomic island

¹Adapted from Malachowa *et al.*, (2010).

²PhLOPS_A: resistance to *phenicols*, *lincosamides*, *oxazolidinones*, *pleuromutilins*, and *streptogramin A* compounds.

Abbreviations; MLS, Macrolide, lincosamides and streptogramin B; ACME, arginine catabolic mobile element; PI, Pathogenicity island; SCC, Staphylococcal Cassette Chromosome.

but also an increased susceptibility to elevated temperatures and a five-fold decrease in virulence in a mouse infection model compared to wild type.

1.4.3-Virulence factors

The success of many *S. aureus* strains can be attributed, at least in part, to their ability to acquire and express multiple virulence genes, some of which form part of the accessory genome present on MGEs (Table 1.2), whilst others make up part of the core genome. Certain core-encoded virulence genes are found in virtually all *S. aureus* isolates (Peacock *et al.*, 2002). These virulence factors include cell surface proteins such as the capsular polysaccharide which impedes phagocytosis by neutrophils and has been implicated in enhanced colonisation. Other cell surface virulence factors include the microbial surface components recognising adhesive matrix materials (MSCRAMMS) such as bone binding protein, clumping factors A/B and fibronectin binding proteins A/B; (Fowler *et al.*, 2000; O’Riordan & Lee 2004). The core genome also encodes toxins such as the cytolytic alpha-toxin, coagulase genes, the intercellular adhesion cluster involved in biofilm formation and staphylococcal protein A (*spa*).

Many of the *S. aureus* resistance and virulence genes are located on MGEs that can be horizontally transferred between *S. aureus* strains and from other staphylococcal species (Table 1.2). Much of the genetic variation between *S. aureus* strains is due to the presence of various MGEs such as bacteriophages, SCC/SCC*mec* elements, pathogenicity islands, transposons, insertion sequences and plasmids (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Lindsay & Holden, 2004).

One of the most important MGE-encoded virulence factors are the superantigens which play a key role in the pathogenesis of *S. aureus* disease as they are capable of activating 5-30% of non-specific T cells inducing a massive cytokine storm and notable superantigens in *S. aureus* include the staphylococcal enterotoxin A (encoded by *sea*) and Toxic Shock Syndrome toxin TSS (encoded by *tst*) (Bernal *et al.*, 1999). The evasion of host immune response by *S. aureus* is facilitated by a trio of phage-encoded genes staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*) and staphylococcal complement inhibitor (*scn*) (van Wamel *et al.*, 2006). Recently a virulence determinant, *sasX*, encoded by a bacteriophage has been identified and shown to be associated with the enhanced spreading of MRSA in Asia (Li *et al.*, 2012). *Staphylococcus aureus* isolates also secrete a wide variety of cytotoxins which form pores in the membranes of target cells and lead

to cell lysis and inflammation; these include the phenol soluble modulins; β -toxin (also known as β -haemolysin) and the leukocidins including PVL.

1.4.3.1 Pantone Valentine leukocidin

The PVL toxin is a bicomponent β -barrel pore-forming toxin encoded by the *lukF-PV* and *lukS-PV* genes. Pantone and Valentine first associated the toxin with *S. aureus* SSTIs in 1932 (Panton *et al.*, 1932). The *lukF-PV* and *lukS-PV* subunits assemble into pore-forming cytolytic octamers on the surface of susceptible leukocytes, especially neutrophils (Fig. 1.3). There is also evidence to suggest that PVL can inactivate mitochondria culminating in apoptosis and can trigger inflammatory immune responses (Genestier *et al.*, 2005). PVL has been shown to induce dermonecrosis in animal models which support the associations between PVL and *S. aureus* SSTIs (Ward & Turner, 1980; Cribier *et al.*, 1992). The PVL toxin's role in the serious conditions of necrotising pneumonia and fasciitis is more controversial. Mouse models of sepsis have found no differences in strain virulence in the presence or absence of PVL genes (Voyich *et al.*, 2006). PVL deletion mutants of USA300 (ST8-MRSA-IV) and USA400 (ST1-MRSA-IV) strains showed no altered cytotoxicity in *in vitro* studies with human alveolar epithelial cells compared to wild type (Wardenburg *et al.*, 2007). Other studies using murine lung and skin infection models failed to detect the pathogenic function of the toxin in cell culture experiments (Voyich *et al.*, 2006; Bubeck *et al.*, 2007a; Bubeck *et al.*, 2007b). Investigations of acute pneumonia in mice models have suggested PVL as a major virulence factor (Labandeira-Ray *et al.*, 2007). Similarly, rabbit bacteraemia studies have found PVL has a transient effect during the acute phase of infection (Diep *et al.*, 2008). However, evidence suggests that the actions of *S. aureus* toxins are strongly dependent on the species model used so it is possible that animal models are not reflecting the *in vivo* effects of PVL in human infection. In various studies involving different sampling groups *pvl* has been reported in 60-100% of CA-MRSA strains investigated (David & Daum, 2010). Although there is a strong epidemiological link between PVL and the pathogenesis of CA-MRSA strains its true role in the virulence of CA-MRSA has yet to be determined.

As with many virulence genes in *S. aureus*, the PVL genes are encoded on an MGE, in this instance on a bacteriophage (Fig. 1.2 & section 1.4.3.2) and 13 distinct bacteriophages encoding PVL have been identified to date (Kaneko *et al.*, 1998; Narita *et*

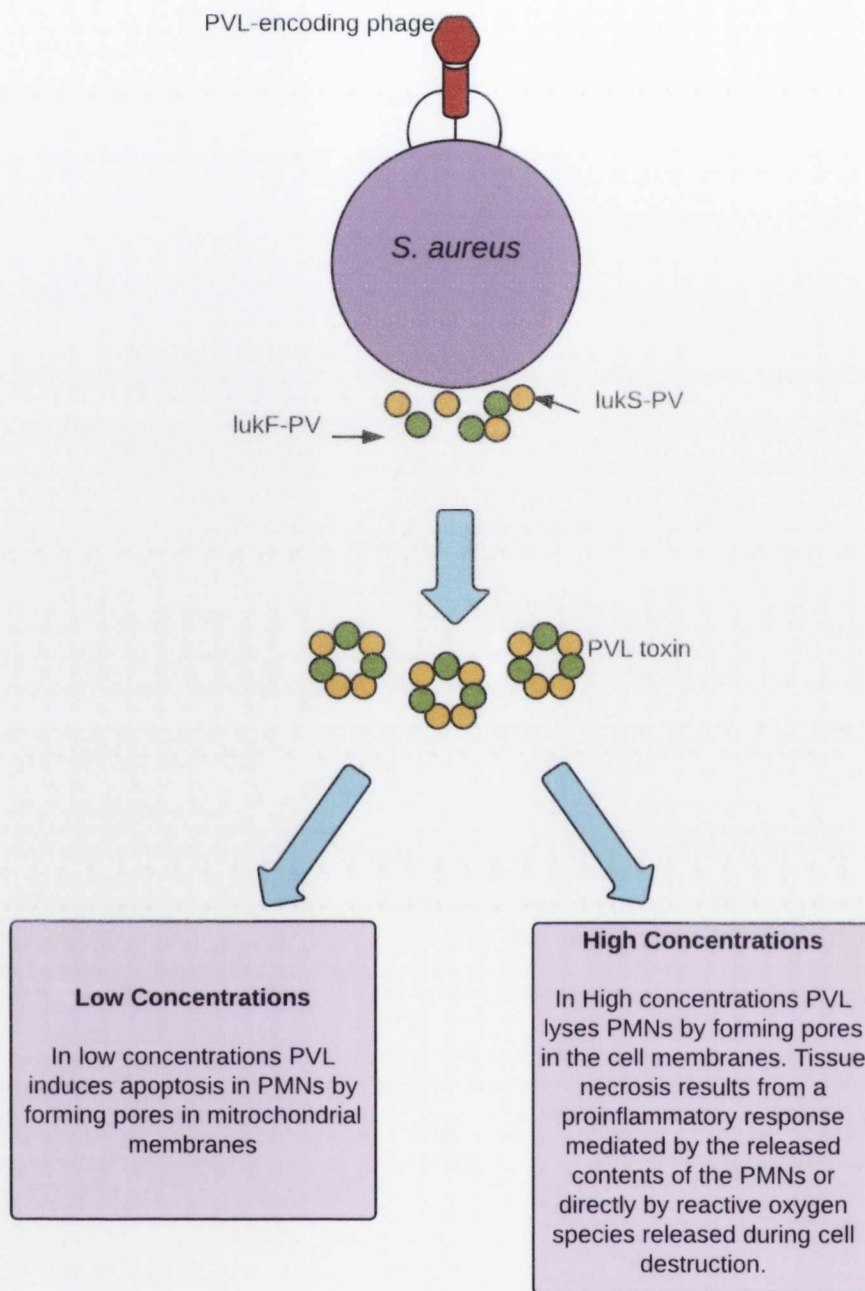


Figure 1.3 The mechanism of Panton-Valentine leukocidin (PVL)-mediated tissue necrosis. Bacteriophage encoding the genes for the two PVL subunits, *lukF-PV* and *lukS-PV*, infect *S. aureus* and form a lysogen with the phage genome stably integrated into the bacterial chromosome. PVL genes are expressed and the two subunits are excreted from the *S. aureus* cell and form a heptameric structure on the surface of polymorphonuclear leukocytes (PMNs). Low concentrations of PVL lead to a novel pathway of apoptosis in PMNs by forming pores in mitochondrial membranes. In higher concentrations lysis of PMNs occurs releasing reactive oxygen species and other proinflammatory markers into the surrounding tissues leading to inflammation and necrosis. Adapted from Boyle-Vavra and Daum (2007).

al., 2001; Ma *et al.*, 2006; Ma *et al.*, 2008; Wirtz *et al.*, 2010; Boakes *et al.*, 2011; Haddad & Moineau, 2013; Prabhakara *et al.*, 2013). New *pvl*-phages are being found around the world with the emergence and spread of new strains, most recently a novel *pvl*-phage was discovered in ST772 which encoded both *pvl* and *sea* (Prabhakara *et al.* 2013).

1.4.3.2 Bacteriophages

Bacteriophages play an important role in *S. aureus* pathogenicity encoding virulence factors such as the toxins PVL, enterotoxin A (*sea*) and exfoliative toxins (*etA*) and immune evasion genes (*sak*, *chp* and *scn*). Bacteriophage can undergo one of two reproductive cycles, lysogenic or lytic. Lysogenic bacteriophages insert their DNA directly into the host chromosome where it is known as a prophage. Subsequent replication of host cells transfers the prophage to the host cell progeny. During the lytic cycle the phage DNA is inserted into the host cell where it circularises but does not integrate into the chromosome, instead it utilises the host cell to replicate and generate new phage particles that lyse the host cell and are released. Phage which can replicate using both lysogenic and lytic cycles are known as temperate phage.

Bacteriophage are found widespread among *S. aureus* and have been studied extensively (Kwan *et al.*, 2005; Lindsay *et al.*, 2010). Initially they were used to type clinical *S. aureus* isolates (Wentworth, 1963; Rosenblum, 1964). All *S. aureus* studied to date carry at least one lysogenic phage with many carrying up to four (Lindsay, 2010). Over 80 different staphylococcal bacteriophage sequences have been published, the majority of which belong to the *Siphoviridae* (Goerke *et al.*, 2009). Typically *S. aureus* phage are temperate phage. The *Siphoviridae* consist of icosahedral capsids which can be elongated or isometric and non-contractile tails which can be short (130 nm) or long (400 nm) (Ackermann, 1998). The size of *S. aureus* phage ranges from 20 kb to 125 kb (Kwan *et al.*, 2005). Typically *S. aureus* phage comprise six functional modules: lysogeny, replication, transcriptional regulation, packaging head and tail and lysis (Brussow & Desiere, 2001). A common feature of many *S. aureus* phage is the location of toxin genes between the phage lysin, involved in cell wall cleavage during the lytic cycle, and the right attachment site (*attR*). The PVL, enterotoxin A, exfoliative toxin A and staphylokinase genes are all located beside the *attR* sites of various phage (Coleman *et*

al., 1989; Kaneko *et al.*, 1998; Narita *et al.*, 2001). Recombination events between phage and between phage and host are common (Kwan *et al.*, 2005; Wirtz *et al.*, 2010).

These phages provide *S. aureus* with additional genes that enhance the isolate's virulence, a phenomenon known as phage conversion. Positive lysogenic conversion has been described for the genes encoding Pantone-Valentine leukocidin (*lukF/S-PV*) (Kaneko *et al.*, 1998), the cell-wall anchored protein SasX (Li *et al.*, 2012) and exfoliative toxin A (Yamaguchi *et al.*, 2001). Up to five virulence factors have been found to be encoded by a single phage. Phage phiN315, a phiSa3 family phage, encodes five genes forming part of the IEC including enterotoxin S (*sea*), staphylokinase (*sak*), the chemotaxis inhibitory protein (*chp*) and the staphylococcal complement inhibitor (*scn*) (Coleman *et al.*, 1989; Goerke *et al.*, 2006; vanWamel *et al.*, 2006). Negative conversion occurs where chromosomal factors are disrupted through phage integration and can be seen in phiSa3 phage which integrate into the β -haemolysin gene *hly* producing lysogens defective for β -haemolysin but which encode IEC (Carroll *et al.*, 1993). Phage integration is site- and orientation-specific and specificity is defined by phage and host attachment site sequences and mediated by integrase (Carroll *et al.*, 1995). Phage expression is detectable during lysogeny but maximised upon entry to the lytic cycle where latent promoters are activated (Sumby & Waldor 2003; Goerke *et al.*, 2006). Phage conversion can often be linked to specific disease outcomes.

Bacteriophage have been investigated as a potential therapy against *S. aureus* infections (Kazmierczak *et al.*, 2014; Woo & Ahn, 2014) and as a tool for fighting biofilm development (Parasion *et al.*, 2014).

1.5 Typing of *S. aureus*

Typing of any microorganism aims to differentiate between strains among groups of isolates, using either phenotypic or genotypic methods. The development of methods for typing of *S. aureus* has enhanced our understanding of its evolution and spread and plays an important role in monitoring and preventing the spread of *S. aureus* infections.

An extensive variety of typing techniques, both phenotypic and genotypic, have been developed for *S. aureus*. Phenotypic methods include antimicrobial resistance typing and biotyping but these have largely been replaced by genotypic methods such as pulsed-field gel electrophoresis (PFGE), DNA microarray profiling and DNA-sequencing based

typed methods such as MLST and *spa* typing (Tenover *et al.*, 1995; Enright *et al.*, 2000; Monecke *et al.*, 2007) and even more recently by whole-genome sequencing (WGS) (Holden *et al.*, 2013). For outbreak investigations a highly discriminatory typing method is necessary, but for longer-term studies a more stable marker is required so that similarities between isolates can still be identified.

1.5.1 Pulsed-field gel electrophoresis (PFGE)

During the 1990's the gold standard for typing of *S. aureus*, particularly in outbreak situations, was PFGE, which uses rare cutting restriction endonuclease enzymes such as *Sma*I to digest whole-genomic DNA from isolates followed by fragment separation by PFGE (Tenover *et al.*, 1995). Following PFGE the resulting banding patterns are analysed using computer based software. Similarities can be calculated using an unweighted paired group method utilising the Dice coefficient and arithmetic averages (UPGMA) and pattern differences can be interpreted using criteria as described by Tenover *et al.* (1995). The PFGE method is highly discriminatory and detects uncharacterised genomic variation including point mutations, insertions and deletions which accumulate rapidly and therefore is more suited to outbreak investigations. It is also labour intensive and time consuming and requires the comparison and often subjective interpretation of complex banding patterns. During the last decade, there has been a move away from PFGE towards more recently developing DNA sequencing-based typing methods including *spa* typing (for MRSA and MSSA) (Shopsin *et al.*, 1999), *dru* typing (for MRSA only) (Goering *et al.*, 2008) and MLST (for MRSA and MSSA) (Enright *et al.*, 2000). These methods provide a more accurate, reproducible and non subjective means of typing isolates allowing for data comparison between laboratories.

1.5.2 Multilocus sequence typing (MLST)

Staphylococcus aureus MLST involves the sequencing of internal fragments of seven housekeeping genes i.e. *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*. Due to the relative stability of these seven genes, MLST is useful for determining the clonal relatedness of isolates over long periods of time but lacks the discrimination required for outbreak investigations (Enright *et al.*, 2010). Isolates with identical allele sequences in all seven of the genes are assigned to a given sequence type (ST), STs which have identical allelic sequences at five of the seven genes are considered closely related and are assigned to a clonal complex (CC). While MLST can be expensive because segments of seven genes

have to be sequenced it results in unambiguous DNA sequence data that can easily be compared between users. For *S. aureus*, both MRSA and MSSA, MLST has been widely used, and has provided insights into its epidemiology and evolution both at local and international levels (Shore *et al.*, 2012; Rodriguez *et al.*, 2015). In addition, the resulting ST is used as part of the internationally accepted strain nomenclature for *S. aureus* genotypes along with the SCCmec type for MRSA isolates (Table 1.1) (IWG SCC 2009).

1.5.3 SCCmec typing

In order to comprehensively characterise MRSA it is essential that not only the genetic background but also the SCCmec element is examined and characterised. Isolates within the same genetic background i.e. ST, can harbour different SCCmec types. The SCCmec type of an isolate is defined by the different combinations of the *mec* and *ccr* gene complexes that it harbours and J regions define subtypes (Fig. 1.4). Multiplex PCRs can be performed in order to correctly identify the SCCmec type and subtype of an isolate (Oliveira *et al.*, 2002; Kondo *et al.*, 2007; Milheirco *et al.*, 2007). However, it can be time consuming and expensive to perform several multiplex PCRs on each isolate to identify the SCCmec type. Also, SCCmec typing PCRs constantly require updating to incorporate newly identified types and subtypes.

1.5.4 spa typing

The *spa* typing method involves the sequencing of a variable repeat region within the *S. aureus* staphylococcal protein A gene *spa*. It can be used for both short and long-term epidemiological studies. It offers enhanced discrimination of isolates compared to MLST and the MLST ST or CC can be inferred from the resulting *spa* type (Shopsin *et al.*, 1999; Tavares *et al.*, 2013) and while it is not as discriminatory as PFGE it is cheaper and easier to perform and interpret and data is more reproducible. Consequently in many centers *spa* typing has now replaced both PFGE and MLST as a routine *S. aureus* typing method. A large study that investigated the dominant clones of *S. aureus* from BSIs in Europe in 2006/2007 utilised *spa* typing to identify significant local, regional and national changes in the prevalence of MRSA and MSSA (Grundmann *et al.*, 2010).

1.5.5 DNA microarrays

Recent advances in sequencing technologies (section 1.5.6) have resulted in the publication of an extensive range of the whole-genome sequences of *S. aureus* strains.

Type I(1B)
(NCTC10442)

Type II(2A)
(N315)

Type III(3A)
(85/2082)

Type IV(2B)
(CA05)

Type IV(2B&5)
(ZH47)

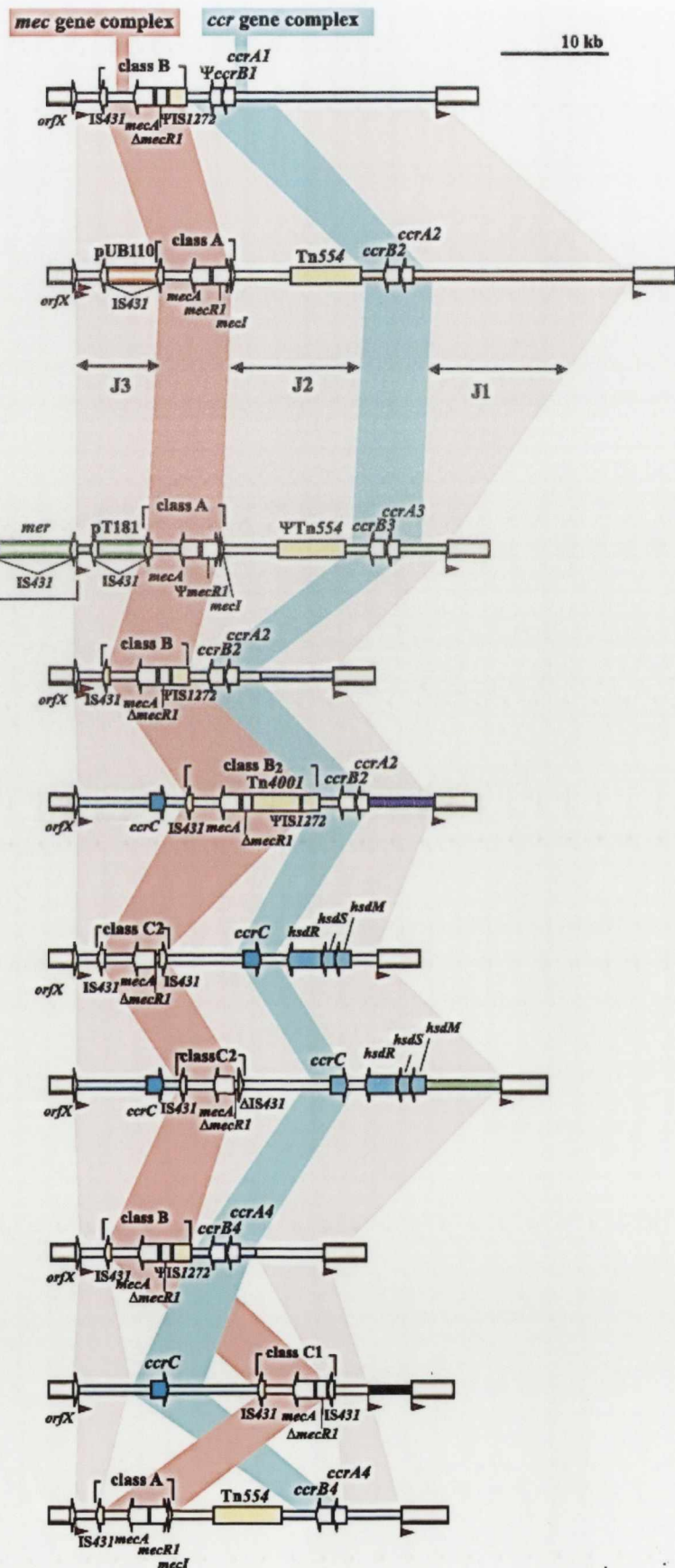
Type V(5C2)
(WIS)

Type V(5C2&5)
(TSGH17, PM1)

Type VI(4B)
(HDE288)

Type VII(5C1)
(JCSC6082)

Type VIII(4A)
(C10682)



These sequences have been analysed and compared to facilitate the development of high-throughput DNA microarrays. Among these Monecke *et al.*, (2007, 2008) developed a diagnostic DNA microarray (Staphy-Type Kit) capable of the simultaneous detection of 336 gene targets including antimicrobial resistance and virulence genes, species-specific markers, SCCmec, toxins, *agr* and capsule genes and their allelic variants. The DNA microarray can be used to screen large numbers of isolates within a relatively short time frame providing detailed information on the virulence and antimicrobial resistance gene content of isolates and on the genetic background of the isolates by assigning them a MLST CC and ST and to a SCCmec type. Several studies have highlighted the effectiveness of this DNA microarray system for the characterisation of MRSA isolates. A study of pandemic *S. aureus* clones utilised DNA microarray profiling to distinguish and provide insights into the virulence and antimicrobial resistance gene content of dominant *S. aureus* clones (Monecke *et al.*, 2011). There is also excellent correlation between MLST and SCCmec typing and the DNA microarray and also between phenotypic antimicrobial resistance and gene content (Shore *et al.*, 2012).

1.5.6 Whole-genome sequencing

A recent molecular technique with huge transformative potential for clinical diagnostics and outbreak management is whole-genome sequencing (WGS). Early sequencing techniques involved the chain-termination or Sanger sequencing method which was originally developed in 1977. It utilised a sequencing-by-synthesis approach where a DNA-dependent polymerase was used to synthesise a complementary strand of the single-stranded DNA template (Sanger *et al.*, 1977a). Using this method it was very laborious to sequence an entire genome, taking huge teams of staff and many years to complete the sequencing of a single bacterial genome (Sanger, 1977b). In 2001 the first full *S. aureus* genome was published using the Sanger method (Kuroda *et al.*, 2001). Since then there has been an ever changing sequencing market focusing mainly on two areas, the high throughput platforms such as the Illumina HiSeq (Illumina) and the benchtop sequencers such as the Illumina MiSeq. High throughput technologies have the advantage of lower costs per run, shorter reads reducing discrepancies and high output, however, installation costs are high and many facilities and clinics would not meet the sample number (approximately 250) per run which would make it cost efficient. Next generation sequencing technologies allow the potential for culture-independent antimicrobial susceptibility testing. This is particularly important in the clinical

environment where results are required within a short time frame to benefit patients. The benchtop sequencers are cheaper to install though with a slightly higher cost per run (Fricke & Rasko, 2014). The introduction of nanopore technologies could further change the next generation sequencing market allowing for faster long-read sequencing from low-input samples which are more affordable (Maitra *et al.*, 2012). It is reasonable to assume that the costs associated with WGS will eventually match those of conventional epidemiological typing techniques.

Sequencing approaches have been used to complement and confirm mechanisms of antibiotic resistance in *S. aureus*, confirming the interspecies transfer of *mecA* from *S. epidermidis* to *S. aureus* (Bloemendaal *et al.*, 2010) and the transfer of vancomycin resistance from enterococci to *S. aureus* (Kos *et al.*, 2012). Recent studies have highlighted the potential of WGS within investigations of the global evolution of MRSA strains (Rasko *et al.*, 2011; Holden *et al.*, 2013), for metagenomic sequencing for the detection of infectious disease (Loman *et al.*, 2013), and for the prediction of virulence and resistance (Laabei *et al.*, 2014).

Studies have confirmed the application of WGS for MRSA outbreak and local investigations (Gardy *et al.*, 2011; Eyre *et al.*, 2012; Koser *et al.*, 2012; Harris *et al.*, 2013). The evolutionary rate of *S. aureus* is estimated to be in the order of 10^{-6} substitutions/site/year (Harris *et al.*, 2010; Nubel *et al.*, 2010) though this has been debated and may be clone specific. The analysis of single nucleotide polymorphisms (SNPs) across the entire genome allows for the differentiation of closely related isolates. It has provided new insights into the emergence and transmission of MRSA strains (Harris *et al.*, 2010; Harris *et al.*, 2013; Köser *et al.*, 2012; McAdam *et al.*, 2012).

1.6 MRSA and MSSA in Ireland

There have been significant changes in the predominant clones of MRSA circulating in Irish hospitals over the last 40 years (Shore *et al.*, 2005) (Figure 1.2). The ST250-MRSA-I/I-*pIs* clone which predominated in the 1970's and early 1980's was replaced by ST239-MRSA-III/III-*pI258/Tn554* in the 1980s. This clone was in turn displaced by ST8-MRSA-IIA-E in the 1990's and finally in 2002 ST22-MRSA-IV became the dominant clone. The ST22-MRSA-IV clone now accounts for approximately 80% of HCA-MRSA

in Ireland each year (NMRSARL, 2012). The reasons for MRSA strain replacements are not well understood as discussed in section 1.4 above.

While ST22-MRSA-IV has remained the predominant clone in Irish hospitals for a decade, the proportion of MRSA BSIs in Ireland have reduced significantly in recent years from 41.9% in 2006 to 20.3% in 2013 (Fig. 1.1) while the proportion of MSSA BSIs have increased from 58% in 2006 to 79.7% in 2013. *Staphylococcus aureus* account for 20.99% of all BSIs identified in Ireland (HPSC, 2014). With the large numbers of circulating MRSA and MSSA strains at any one time it is likely that strains are in competition with each other, with the fitter strains replacing less well adapted ones. An investigation into sporadically-occurring MRSA strains identified in Irish hospitals between 2000 and 2012 identified a possible reservoir of new virulent and multidrug resistant emerging clones, which could replace the ST22-MRSA-IV clone (Kinnevey *et al.*, 2014). Of the 88 isolates identified almost 50% harboured genes for two or more MGE-encoded virulence factors and resistance to two or more antimicrobial classes. However, the ST22-MRSA-IV clone could also be undergoing clonal decline, where for reasons unknown they are no longer thriving in a specific area and are being outcompeted by MSSA. Little is known about the circulating strains of MSSA in Ireland despite its increasing importance amongst BSIs (HPSC, 2014). A pan-European study investigating MRSA and MSSA causing BSIs identified 84 MSSA isolates during a six month period in Ireland. A total of 54 *spa* types were found among the isolates, the majority of which were represented by one to three isolates only showing a highly diverse MSSA population (Grundman *et al.*, 2010).

PVL-positive MRSA were first reported in Ireland between 2002 and 2005 (Rossney *et al.*, 2007). Six different clones were identified by MLST and SCCmec typing, with ST30-MRSA-IV and ST8-MRSA-IV prevailing. It was speculated that the diversity of genotypes identified was the result of the importation of different strains. However, despite the increasing prevalence of PVL-positive/CA-MRSA worldwide, there have been no more studies to determine the genotypes of PVL-positive MRSA since 2005, no studies of PVL-positive MSSA in Ireland and no detailed studies of the virulence and antimicrobial resistance genes present in *pvl*-positive *S. aureus* isolates in Ireland.

Since 2002 ongoing epidemiological surveillance of prevalent and emerging *S. aureus* clones in Ireland is routinely undertaken by the National MRSA reference laboratory

(NMRSARL) at St. James's Hospital, Dublin. At the NMRSARL epidemiological typing of MRSA isolates is performed using antibiogram resistogram (AR) typing, PFGE, *spa* typing and an in-house multiplex PCR for *mecA*, *mecC*, *nuc* and *lukF/S-pvl*. Data are also collected as part of the European Antimicrobial Resistance Surveillance System (EARS-Net), which monitors antimicrobial resistance among seven pathogens causing bloodstream infections in Europe, including *S. aureus*.

1.7 Aims of the present study

1. Despite a global increase in *pvl*-positive *S. aureus* isolates causing serious and often fatal infections in otherwise healthy individuals in the community there have been no published data on the overall prevalence and molecular epidemiological characteristics of *pvl*-positive MRSA in Ireland since 2005, no data on *pvl*-positive MSSA, and the isolates that were previously examined were not extensively investigated for virulence and antimicrobial resistance genes. **Therefore, the first aim of the present study was to investigate the clinical and molecular epidemiology of *pvl*-positive MRSA and MSSA identified by the NMRSARL between 2002 and 2012.**

2. With the emergence and spread of MRSA strains carrying new *pvl*-encoding bacteriophages worldwide and the potential for increased virulence of these strains **the second aim of the present study was to characterise the genetic organisation of *pvl*-encoding phage genomes from a selection of *pvl*-positive MRSA isolates representative of the dominant *pvl*-positive MRSA clones identified in Ireland between 2002 and 2012.**

3. The reasons for the decline of MRSA among *S. aureus* BSIs and the increase in numbers of MSSA BSIs remain unclear. **The third part of this study aimed to comprehensively characterise and compare the molecular and epidemiological characteristics of MRSA and MSSA causing BSIs among patients in Irish hospitals during two different time periods when MRSA BSIs were at their peak (2006/07) and when MRSA BSIs had declined and MSSA BSIs increased (2011) in order to identify if any change in virulence factor(s), antimicrobial resistance gene(s), clonal type or patient demographics could be attributed to the decline in MRSA BSIs.**

4. A further investigation of the decline of MRSA among *S. aureus* BSIs was also performed using growth curves and whole-genome sequencing **with the aim of investigating if the clonal decline of ST22-MRSA-IV may have contributed to the decline in the incidence of MRSA BSIs in Ireland in recent years.**

Chapter 2

Materials and Methods

2.1 Bacterial isolates

Details of the *S. aureus* isolates investigated in the present study are shown in Table 2.1. This included 283 *pvl*-positive MRSA and MSSA recovered in Ireland between 2002 and 2012 and 402 MRSA and MSSA isolates recovered from patients with BSIs in 2006/07, 2009 and 2011.

2.2 General microbiological methods

2.2.1 Bacterial storage and culturing

All *S. aureus* isolates were cultured on Columbia Blood agar (CBA) (Fannin Ltd., Dublin, Ireland) or Oxoid Brain Heart Infusion (BHI) agar plates (Thermo Scientific, City, Country.) and were incubated overnight at 37°C in a static incubator (Gallenkamp, Leicester, UK). Isolates were stored at -70°C on plastic cryogenic beads in individual preserver vials (Protect Bacterial Preservation System, Technical Services Ltd., UK). Isolates were cultured by removing a single bead from the storage vial using a sterile inoculating loop and inoculating onto a CBA plate. Plates were incubated overnight at 37°C in a static incubator.

2.2.2 Chemicals, oligonucleotides and enzymes

All chemicals used were of analytical grade or molecular biology grade and were purchased from the Sigma-Aldrich Chemical Co. (Tallaght, Dublin, Ireland). All oligonucleotides were custom synthesised by Sigma-Aldrich and stored as stock solutions of 10 mM at -20°C. DNA loading dye and molecular weight markers (100 bp and 1 kb) were purchased from the Promega Corporation (Madison, Wisconsin, USA). The DNA loading dye was diluted to a 1 X working stock for use. Proteinase K (20 mg/ml) (Sigma-Aldrich) lysozyme (25 mg/ml) (Sigma-Aldrich) and lysostaphin (1 mg/ml) (Sigma-Aldrich) were prepared using molecular grade biology reagent water (Sigma-Aldrich).

2.2.3 Water, buffers and solutions

The Milli Q Biocel system (Millipore Ireland, Carrigtwohill, Ireland) was used to provide ultra-pure water which was used in the preparation of buffers and agarose gels. Molecular biology reagent water (Sigma-Aldrich) was used in all PCR reactions, DNA elutions and dilutions. Tris-borate/EDTA (TBE) at a 0.5 X concentration was used as the

electrophoresis buffer for agarose gel electrophoresis. This was initially prepared at a 10 X concentration and consisted of 0.45 M trizma base, 0.45 M boric acid and 0.01 M EDTA, pH 8. This was diluted using ultra-pure water to a 0.5 X working stock.

2.3 Isolate identification and antimicrobial susceptibility testing

2.3.1 Identification of isolates as *S. aureus*

All isolates were identified as *S. aureus* at the NMRSARL by the detection of staphylocoagulase using the tube coagulase test (Rossney *et al.*, 2006). Inconclusive results were further tested using the Pastorex latex agglutination kit (PastorexTM, Staph-Plus Kit, Bio-Rad, Marnes la Coquette, France) and by detection of thermostable deoxyribonucleases (Rossney *et al.*, 1994). If necessary, isolates were further subjected to biochemical identification using API Staph identification strips (BioMérieux, sa, Marcy-l'Etoile, France) according to the manufacturer's instructions.

2.3.2 Identification of isolates as methicillin-resistant or methicillin-susceptible

Isolates were tested for susceptibility to methicillin at the NMRSARL using 30 µg cefoxitin discs (Oxoid, Basingstoke, UK) and CLSI methodology and interpretive criteria (Table 2.2), PCR for *mecA* (Rossney *et al.*, 2007), or, if necessary, the MastalexTM-MRSA kit (MAST Group, Bootle, Merseyside, UK) which detects PBP2a production and was used according to the manufacturer's instructions.

2.3.3 Antimicrobial susceptibility testing

The susceptibility of all isolates to 23 antimicrobial agents used in the AR typing panel was determined by disk diffusion (Rossney *et al.*, 1994; Rossney *et al.*, 2007). The antimicrobial agents tested together with the disk concentrations and breakpoints used are shown in Table 2.2. Susceptibility testing was performed in accordance with the CLSI methodology and where available, interpretive criteria (CLSI 2006). For agents not listed under CLSI guidelines, the disk concentrations and interpretive criteria of Rossney *et al.* (2007) were used. All antimicrobial susceptibility testing was carried out at the NMRSARL, Dublin, Ireland.

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

Chapter	Purpose of the study and source of isolates	Years of isolation	Number of isolates	
			MRSA	MSSA
3	Molecular characterisation of 283 <i>pvl</i> -positive <i>S. aureus</i> isolates recovered from patients in Ireland ¹	2002-2012	234	49
4	Whole-genome sequencing of 12 <i>pvl</i> -positive <i>S. aureus</i> isolates representative of the three main genotypes of <i>pvl</i> -positive MRSA identified in Chapter 3 to determine the genetic organisation of the <i>pvl</i> phage carried by each isolate	2008-2012	12	N/A ³
5	Comparative molecular analysis of 354 <i>S. aureus</i> isolates recovered from patients with bloodstream infections during two six-month periods ²	2006/07	83	84
		2011	73	114
6	Comparative analysis of ST22-MRSA-IV isolates	2006	24	N/A
		2009	24	N/A
		2011	20	N/A

¹Twenty-five of the *pvl*-positive MRSA isolates have been described previously (Rossney *et al.*, 2007).

²The 167 *S. aureus* isolates recovered in 2006 have also been described previously (Deasy, 2014; Grundmann *et al.*, 2010).

³N/A, not applicable as no isolates were investigated.

Table 2.2 Antimicrobial agents used and the breakpoints used for susceptibility testing of *S. aureus* isolates investigated in the present study

Antimicrobial agent	Antimicrobial agent abbreviation	Disk concentration (µg/disk)	Zone breakpoints (mm) ¹			Reference for interpretive criteria
			R ² (<)	I ²	S ² (≥)	
Amikacin	Ak	30	14	15-19	20	CLSI, 2009
Ampicillin	Ap	10	28	None ³	29	CLSI, 2009
Cadmium acetate	Cd	130	10	11-15	16	Rossney <i>et al.</i> , 2007
Cefoxitin	Fox	30	24	None ³	25	CLSI, 2013
Chloramphenicol	Cl	30	12	13-17	18	CLSI, 2009
Ciprofloxacin	Cp	5	15	16-20	21	CLSI, 2009
Erythromycin	Er	15	13	14-22	23	CLSI, 2009
Ethidium bromide	Eb	60	13	14	15	Rossney <i>et al.</i> , 2007
Fusidic acid	Fd	10	23	24-26	27	CLSI, 2009
Gentamicin	Gn	10	12	13-14	15	CLSI, 2009
Kanamycin	Kn	30	13	14-17	18	CLSI, 2009
Lincomycin	Ln	2	14	15-16	17	Rossney <i>et al.</i> , 2007
Mercuric chloride	Mc	10	13	14	15	Rossney <i>et al.</i> , 2007
Mupirocin	Mp	5	12	13-19	20	CLSI 2009
Mupirocin	Mp	200	17	18-29	30	CLSI, 2009
Neomycin	Nm	30	15	16-29	30	Rossney <i>et al.</i> , 2007
Phenyl mercuric acetate	Pma	10	24	25-28	29	Rossney <i>et al.</i> , 2007
Rifampicin	Rf	5	16	17-19	20	CLSI, 2009
Spectinomycin	Sp	500	13	14-19	20	Rossney <i>et al.</i> , 2007

Continued overleaf

Antimicrobial agent	Antimicrobial agent abbreviation	Disk concentration (µg/disk)	Zone breakpoints (mm) ¹			Reference for interpretive criteria
			R ² (<)	I ²	S ² (≥)	
Streptomycin	St	25	13	14-15	16	Rossney <i>et al.</i> , 2007
Sulphonamide	Sn	300	12	13-16	17	CLSI, 2009
Tetracycline	Te	30	14	15-18	19	CLSI, 2009
Tobramycin	Tb	10	17	18	19	CLSI, 2009
Trimethoprim	Tp	5	10	11-15	16	CLSI, 2009
Vancomycin	Vn	30	14	None	15	CLSI, 2009

¹Diameter of zones of growth inhibition around antimicrobial impregnated discs at the concentrations shown were recorded in mm.

²Abbreviations: R, resistant; I, intermediate resistance; S, susceptible.

³No intermediate breakpoint.

2.4 Molecular Methods

2.4.1 Extraction of whole-genomic DNA

The following method was used for DNA extraction for all applications except for the confirmation of the presence or absence of specific antimicrobial resistance genes (see below). A pure culture of each isolate was obtained by plating out individual storage beads onto CBA plates and incubating overnight at 37°C in a static incubator. A single colony was then lawned onto one half of a fresh CBA plate using a sterile inoculating loop and again incubated overnight at 37°C in a static incubator. For cell lysis, culture growth from a 2.5 cm² area was added to 200 µl of lysis buffer and lysis enhancer (supplied with the DNA microarray StaphType Kit, Alere, Jena, Germany) (see section 2.4.4 below) and incubated for 30-60 min at 37°C. Proteins were then removed by the addition of 25 µl of proteinase K and 200 µl of buffer AL (both supplied with the Qiagen DNeasy Blood and Tissue kit Qiagen, Crawley, UK) and a further incubation step for 30 min at 70°C (static incubation). The Qiagen DNeasy Blood and Tissue kit was then used to extract total genomic DNA from each isolate according to the manufacturer's instructions. In brief, following protein digestion, the free DNA was then bound to the mini-column supplied with the DNeasy Blood and Tissue kit which contains a silica-gel membrane that selectively binds free DNA. A series of washes were then undertaken with buffers supplied with the kit. The buffers contained ethanol and salt to clear cellular debris from the DNA bound to the membrane. The DNA was then eluted using 50 µl of Sigma molecular biology-grade water and 10 min incubation at room temperature followed by centrifugation at 6,000 x g for 1 min. Samples were then concentrated by heating at 70°C for 30 min with microfuge tube lids open. A 3 µl aliquot of diluted extracted DNA (1/50) was then electrophoresed in a 0.8% (w/v) agarose gel to ensure the absence of RNA in each sample and to assess DNA quality. The concentration of DNA was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). DNA samples were then stored in the short term at -4°C and at -20°C for the long term.

The following method was used to isolate DNA for immediate use in PCRs to confirm the presence or absence of antimicrobial resistance genes (section 2.4.3.). Isolates stored on Protect beads were lawned onto CBA plates and cultured overnight in a static incubator at 37°C. A loopful of the overnight growth was then transferred to 100 µl of

Sigma sterile water in a 1.5 ml Eppendorf microfuge tube (Eppendorf, Hamburg, Germany). This was then boiled at 100°C for 10 min in a water bath. The sample was then centrifuged at 16,000 rpm for five min. The resulting supernatant was transferred to a fresh 1.5 ml Eppendorf tube and stored at 4°C for immediate use.

2.4.2 Polymerase chain reaction (PCR) and DNA sequence analysis

All PCRs (unless otherwise stated in the relevant chapters) were performed using Go Taq DNA polymerase (Promega) according to the manufacturer's instructions. All PCR products were visualised by agarose gel electrophoresis and then purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Sequencing of target genes was carried out commercially by Geneservice (SourceBioscience, Guinness Enterprise Centre, Dublin and Tramore, Co Waterford), using Sanger sequencing, with an ABI 3730x1 DNA analyser with ABI BigDye terminator v3.1 cycle sequencing kit. The resulting DNA sequences were analysed using the BioNumerics (versions 5.1-7.5: Applied Maths, Ghent, Belgium) or DNA strider (version 1.3f11, CEA Saclay, Gif-sur-Yvette, France) software packages unless otherwise indicated. BLAST software (<http://ncbi.nih.gov/BLAST>) was used to perform homology searches. Allignment of sequences was performed using CLUSTALW (EMBL-EBI, Cambridge, UK) and Artemis (Sanger, UK).

2.4.3 PCR and sequencing of antimicrobial resistance genes

For some isolates the antimicrobial resistance phenotype did not correlate with the antimicrobial resistance genes detected using DNA microarray profiling (section 2.4.4). In these instances, further PCRs were carried out to confirm the presence or absence of specific antimicrobial resistance genes. This included PCR detection of *aacA-aphD*, *aadD*, *qacA*, *qacC*, *blaZ*, *lnu(A)*, *msr(A)*, *mph(C)*, *ileS2*, *tet(M)*, *tet(K)* and *dfrS1*. The primers used are shown in Table 2.3.

2.4.3 *spa* typing

spa typing was performed as described previously (Shore *et al.*, 2008) using the primers and PCR conditions recommended by the European Network of Laboratories for Sequence Based Typing (SeqNet) (<http://www.segnet.org/>) [1113F (5'-TAAAGACGATCCTTCGGTGAGC-3') and 1514R (5'-CAGCAGTAGTGCCGTTTGCTT-3')]. Thermal cycling conditions were as follows:

Table 2.3 Primers used in the present study to confirm the presence or absence of antimicrobial resistance genes where the antimicrobial resistance phenotype did not correlate with the genotype

Class of antimicrobial agents	Resistance gene	Relevant resistance phenotype detected ¹	Primer sequence 5' - 3'	GenBank accession no.
Aminoglycosides	<i>aacA-aphD</i>	Ak, Gn, Kn, Tb	F-TATACAGAGCCTTGGGAAGATG R-CCTCGTGTAATTCATGTTCTGGCA	AB246921.1
	<i>aadD</i>	Ak, Kn, Nm, Tb	F-TAGCTGAATAAGAACGGTGCTCTC R-CAC TTCCACCTTCCACTCACC	AB037420.1
	<i>aphA3</i>	Kn, Nm	F-TGAGAATATCACCGGAATTGAA R-GCACTCCGCATACAGCTCGATA	AB300568.1
Antiseptics & disinfectants	<i>qacA</i>	Eb	F-GATCGCACGGTCTATAAGGATT R-CGAGGCCAAATAAAGCAAATCC	AB255366.1
	<i>qacC</i>	Eb	F-CTTAAATCTTCAGAAGGCTTTTCAA R-CGAAACTACGCCGACTATGATTAA	AB125342.1
Beta-lactams	<i>blaZ</i>	Ap	F-TTCAAACAGTTCACATGCCAAAGAG R-AGAACCGTTTGCTGTATTATCAC	AB074882.1
Lincosamides	<i>lnu(A)</i>	Ln	F-AAGTTGAGCTTCTTTGGAAATGC R-ACTCATTGGTTAGATGGAGGC	AM184101.1
Macrolides	<i>msr(A)</i>	Er	F-GCACTTATTGGAGGTAATGGTACTGGC R-TGACGTTGTTGTTCTAACTGTTCTT	AB013298.1
	<i>mph(C)</i>	Er	F-CGAGTGTTTCAGCTAATGTGTTAAT R-ATCAATTACACATCCAACCTCAAAC	AB013298.1
Mupirocin	<i>ileS2</i>	Mp	F-ACGAAATAAGTGATACTCTAGGAG R-AGTCCATGTCAACCCAGTATCCT	CP000258.1
Tetracycline	<i>tet(M)</i>	Te	F-AGTGGAGCGATTACAGAA R-CATATGTCCTGGCGTGTCTA	X56353
	<i>tet(K)</i>	Te	F-GTAGCGACAATAGGTAATAGT R-GTAGTGACAATAAACCTCCTA	S67449
Trimethoprim	<i>dhfrS1</i>	Tp	F-GTCGCTCACGATAAAACAAAGAGT R-TACGTCTATTTGGCAATGGCTTCCC	AB049452.1

¹ Ak, amikacin; Ap, ampicillin; Eb, ethidium bromide; Er, erythromycin; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Nm, neomycin; Mp, mupirocin; Te, tetracycline; Tp, trimethoprim.

denaturation at 80°C for five min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 90 s. This was followed by a final extension at 72°C for 10 min. The Ridom StaphType software program, version 1.3 (Ridom, GmbH, Wurzburg, Germany) was used for *spa* sequence analysis and assignment of *spa* types.

2.4.4 DNA microarray analysis

Whole-genomic DNA, extracted as described in section 2.4.1, was used for DNA microarray profiling analysis of all isolates. The DNA microarray procedure was performed according to the manufacturer's instructions using the Staph-Type Kit (Alere) and as described previously (Monecke *et al.*, 2008). The arrays consist of individual microarray chips mounted in 8-well microtitre strips. Each chip contains 336 probes, the majority printed in duplicate spots, for genetic markers including species-specific markers, typing markers, antimicrobial resistance genes, genes encoding MSCRAMMS and virulence-associated genes as well as a biotin staining control. DNA from an isolated *S. aureus* colony (unfragmented and RNA-free) was linearly amplified 40-fold and internally labelled with biotin-dUTP, using a PCR and primer mastermix provided with the Staph-Type Kit and using a G-storm GSI Thermocycler (G-Storm, Somerset, UK). The procedure yields single-stranded DNA products as only one antisense primer per target is used. The biotin-16-dUTP PCR products were then hybridised to the DNA oligonucleotide microarrays by mixing the labelled PCR products (10 µl) with 90 µl hybridisation buffer C1 (supplied with the Staph-Type Kit). This hybridisation mixture was then transferred to individual wells of the microtitre strip (supplied with the kit), capped and incubated at 55°C for one hour with shaking at 550 rpm. Following incubation, the hybridisation mixture was removed completely and three washes were performed using 200 µl buffer C2 (supplied with the Staph-Type kit). A volume of 100 µl of horseradish peroxidase-conjugate (reagent C3 containing streptavidin-HRP and Buffer C4 in 1:100 ratio (Staph-Type Kit)) was then added to each of the wells. This was incubated for a further 10 min at 30°C at 550 rpm. The HRP conjugate solution was then removed completely and the wells were washed once with buffer C5. Once the buffer was completely removed the bound biotin-HRP conjugate was stained using reagent D1 (tetramethylbenzidine) (supplied with the Staph-Type Kit). A 100 µl volume of D1 reagent was then added to each well and left to incubate at room temperature for 5 min. Reagent D1 was then removed and the microarray chips were analysed immediately using

the Arraymate array reader and IconoClust software (Arraymate, Alere) (versions 1.0-2.0). Different versions of the software were used during parts of the present study and the specific versions used are detailed in the relevant sections of each chapter. The most recent version (version 2.0) includes probes to detect *mecC* and SCC*mec* XI. Spot recognition was performed automatically; the software interprets the raw data as positive, negative or ambiguous using a previously described algorithm (Monecke *et al.*, 2008). Breakpoints for the signal strength were defined as the average of each individual signal for the control spots and specific species markers. Values were considered as positive if they are >33% of the average, ambiguous if they are between 25-33% and negative if less than 25%. The overall pattern was analysed by the software for the presence or absence of genes and this pattern was then compared to a database of reference *S. aureus* strain profiles previously characterised by MLST. This comparison allowed the assignment of isolates to MLST CCs and STs and SCC*mec* types.

Chapter 3

***pvl*-positive *S. aureus* in Ireland 2002-2012**

3.1 Introduction

Since the mid 1990's there has been a significant increase in the numbers of community-associated (CA-) MRSA infections in healthy individuals outside of the hospital environment (Adcock *et al.*, 1998; CDC, 1999; Frank *et al.*, 1999; Charlebois *et al.*, 2002; Baggett *et al.*, 2003; CDC, 2003; Buckingham *et al.*, 2004; Dietrich *et al.*, 2004; Fridkin *et al.*, 2005). These patients lack the traditional risk factors for *S. aureus* infections. These CA-MRSA strains are genetically distinct from hospital-associated MRSA, they predominantly cause SSTIs but are also linked to the more serious and life threatening infections such as necrotising pneumonia, necrotising fasciitis and BSIs (Daum & Daum, 2010). The pathogenesis of CA-MRSA has in some studies been attributed to the ability of these organisms to express the Panton-Valentine leukocidin (PVL) toxin (Vandenesch *et al.*, 2003; Boyle-Vavra & Daum, 2007). Panton-Valentine leukocidin-positive MRSA infections have been reported among many different groups particularly those with close contact or in low socioeconomic situations (Witte, 2009).

The PVL toxin is a bicomponent β -barrel pore-forming toxin encoded by the *lukF-PV* and *lukS-PV* genes. The LukF-PV and LukS-PV subunits assemble into pore-forming cytolytic octamers on the surface of susceptible leukocytes, especially neutrophils. There is also evidence to suggest that PVL can inactivate mitochondria culminating in apoptosis and can trigger inflammatory immune responses (Genestier *et al.*, 2005). Panton and Valentine first associated the toxin with SSTIs in 1932 (Panton *et al.*, 1932). While outbreaks of PVL-producing methicillin-susceptible *S. aureus* (MSSA) were reported in the 1950s and 1960s (Robinson *et al.*, 2005) it was in the 1990s when PVL was first reported in newly emerging CA-MRSA strains (Lina *et al.*, 1999; Groom *et al.*, 2001). The genes encoding PVL are collectively designated *pvl* and are located on lysogenic prophages. To date thirteen distinct bacteriophages harbouring *pvl* have been identified (Kaneko *et al.*, 1998; Narita *et al.*, 2001; Ma *et al.*, 2006; Ma *et al.*, 2008; Wirtz *et al.*, 2010; Boakes *et al.*, 2011; Haddad & Moineau, 2013; Prabhakara *et al.*, 2013). In various studies involving different sampling groups *pvl* has been reported in 60-100% of CA-MRSA strains investigated (David & Daum, 2010).

There is conflicting data regarding the role of PVL in the pathogenesis of CA-MRSA infection. The PVL toxin has been associated with an increased risk of infection for colonised individuals, an increased need for surgical intervention of SSTIs, more serious

infections in younger patients without underlying risk factors for both MRSA and MSSA infections and PVL-positive isolates have also emerged as significant pathogens in lung infections among cystic fibrosis patients and other immunocompromised patients (Gillet *et al.*, 2002; Elizur *et al.*, 2007; Molina *et al.*, 2008; Goodrich *et al.*, 2009; Masiuk *et al.*, 2010; Muttaiyah *et al.*, 2010; Boan *et al.*, 2015). However, other studies have found no link between the presence of PVL and disease severity (Peyrani *et al.*, 2011; Zhang *et al.*, 2008). While PVL has been shown to induce dermonecrosis in animal models which support the reported associations between PVL and SSTIs (Ward & Turner, 1980; Cribier *et al.*, 1992) the role of PVL in the serious conditions of necrotising pneumonia and necrotising fasciitis is more controversial. Mouse models of sepsis have found no differences in strain virulence in the presence or absence of PVL genes (Voyich *et al.*, 2006). PVL deletion mutants of USA300 (ST8-MRSA-IV) and USA400 (ST1-MRSA-IV) strains showed no altered cytotoxicity in *in-vitro* studies with human alveolar epithelial cells compared to wild type (Wardenburg *et al.*, 2007). Other studies using murine lung and skin infection models failed to detect the pathogenic function of the toxin in cell culture experiments (Voyich *et al.*, 2006; Bubeck *et al.*, 2007a; Bubeck *et al.*, 2007b). In contrast, investigations of acute pneumonia in mice models have suggested PVL as a major virulence factor (Labandeira-Ray *et al.*, 2007). Similarly rabbit bacteraemia studies have found PVL has a transient effect during the acute phase of infection (Diep *et al.*, 2008). However, evidence suggests that the actions of *S. aureus* toxins are strongly dependent on the species model used so it is possible that animal models do not accurately reflect the *in-vivo* effects of PVL in human infection. It is clear that while not all CA-MRSA produce PVL, the success of some CA-MRSA clones is associated, albeit not exclusively, with PVL (Otto 2013).

A number of other factors have also been implicated in the success of pandemic CA-MRSA including (1) the carriage of the smaller SCCmec elements (predominantly SCCmec IV and V), (2) the carriage of less antibiotic resistance genes, (3) the carriage of specific virulence factors such as the arginine catabolic mobile element (ACME), phenol-soluble modulins α (PSM α) and (4) the increased expression of *agr*, *lukAB*, protein A, and α -toxin (alpha toxin) (Diep & Otto, 2008). PSMs and Agr play a role in the lysis of neutrophils and erythrocytes; α -toxin and Agr in *in vivo* virulence; α -toxin has been implicated in experimental skin infection. The haemolysins and bi-component leukocidins play a role in the activation of pro-inflammatory cascades. A point mutation in *agr* has

been shown as a cause of gene regulation and murine pneumonia phenotypes. Extracellular proteases such as aureolysin and the serine proteases play a role in SSTI; (Chen *et al.*, 2014; Kolar *et al.*, 2013; Vandenesch *et al.*, 2012; Wehrhann *et al.*, 2012).

Originally the emergence of PVL-positive CA-MRSA clones appeared to be continent-specific with ST8-MRSA-IV (USA300) and ST1-MRSA-IV (USA400) being associated with the USA, ST80-MRSA-IV with Europe and ST30-MRSA-IV with Oceania (Rasigade *et al.*, 2010). However, these CA-MRSA clones have subsequently spread worldwide and today different *pvl*-positive clones predominate in different regions, e.g., ST59-MRSA-V_T in Asia, ST30-MRSA-IV in New Zealand, ST93-MRSA-IV in Australia, ST80-MRSA-IV in the Middle East and ST22-MRSA-IV and ST772-MRSA-V in India. ST8-MRSA-IV, USA300, has emerged as the most prevalent and successful pandemic strain causing CA-MRSA infections in the USA and Europe with outbreaks being reported in every continent except Antarctica (Pan *et al.*, 2005; Furtado *et al.*, 2006; King *et al.*, 2006; Hota *et al.*, 2007; Liu *et al.*, 2008). It has also emerged as a significant cause of HCA-MRSA infections in the USA (Klein *et al.*, 2007; McAdam *et al.*, 2012). New multiresistant PVL-positive CA-MRSA clones have also recently emerged including ST772-MRSA-V (Bengal-Bay Clone) that has spread across several continents in a very short period of time.

Recent studies have highlighted the complex and changing epidemiology of *pvl*-positive MRSA, including (1) considerable variation in the prevalence rates of *pvl*-positive MRSA in different regions of the world (Witte, 2009; Coombs *et al.*, 2012), (2) the increasing prevalence and polyclonal population structure of *pvl*-positive MRSA isolates in Europe (Ellington *et al.*, 2010; Monecke *et al.*, 2011; Rolo *et al.*, 2012), (3) the increasing prevalence of ST8-MRSA-IV in Europe and the decreasing prevalence of ST80-MRSA-IV (Rolo *et al.*, 2012), (4) the increasing prevalence of multiresistant *pvl*-positive MRSA (Patel *et al.*, 2013), and (5) the spread of *pvl*-positive MRSA into hospitals (Schlebusch *et al.*, 2010; O'Hara *et al.*, 2012; Patel *et al.*, 2013). Furthermore there has been an increasing frequency of reports of infections associated with *pvl*-positive MSSA (Breurec *et al.*, 2011; Otokunefor *et al.*, 2012) that produce similar clinical presentations as *pvl*-positive MRSA, and the former are a potential reservoir for the emergence of *pvl*-positive MRSA.

Between 1999 and 2005, a prevalence rate of 1.8% was reported for *pvl*-positive MRSA in Ireland and six distinct *pvl*-positive MRSA clones (ST30, ST8, ST22, ST80, ST5 and ST154, all harboring *SCCmec* IV), were identified (Rossney *et al.*, 2007). Evidence reported in that study suggested that many of the *pvl*-positive MRSA clones were imported (Rossney *et al.*, 2007).

In many regions, including Ireland, there are currently no national data on the burden of CA-MRSA infections. However, several relatively small groups of subjects have been studied in detail in other countries including African pygmies, prisoners in jails, Aborigines and Native Americans, all of whom were found to have higher than average carriage rates of *S. aureus* and an increased risk of infection compared to non-indigenous populations (Tong *et al.*, 2009; Ruimy *et al.*, 2010; Schaumburg *et al.*, 2011).

Despite the link between *pvl*-positive *S. aureus* and an increasing burden of *S. aureus*-related infections worldwide there have been no published data on the overall prevalence and molecular epidemiological characteristics of the *pvl*-positive MRSA population in Ireland since 2005 and just a single report of a familial outbreak of *pvl*-positive MSSA in Ireland with no molecular epidemiological typing of the isolates undertaken (Heelan *et al.*, 2012). To address this situation, this part of the present study investigated the clinical and molecular epidemiology and detailed characteristics of *pvl*-positive MRSA and MSSA identified by the Irish NMRSARL between 2002 and 2012.

3.2 Materials and Methods

3.2.1 Bacterial isolates

The NMRSARL investigated 7786 *S. aureus* isolates (MRSA and MSSA) between 2002 and 2012, of which 1832 were examined for the presence of the *lukF/S-PV* genes (*pvl*) (Table 3.1). An isolate was selected for *pvl* investigation if it was recovered from a suspected *pvl*-associated infection or, in the case of MRSA only, if the isolate exhibited an antibiogram-resistogram (AR) pattern and/or pulsed-field group (PFG) (see Chapter 2, section 2.3.4) distinct from that of the commonly identified *pvl*-negative healthcare-associated MRSA clones e.g. AR-PFG 06-01, indicative of ST22-MRSA-IV or AR-PFG 13/14-00, indicative of ST8-MRSA-IIA-E +/-SCC_{MI} (Rossney *et al.*, 2006). Of the 1832 isolates investigated for *pvl* 283 (234 MRSA and 49 MSSA) were *pvl*-positive and were investigated further (Table 3.1). This included 24/25 previously described *pvl*-positive MRSA isolates recovered between 2002 and 2005 (Rossney *et al.*, 2007). One MRSA isolate (E1760), previously reported as *pvl*-positive (Rossney *et al.*, 2007), was excluded from the present study because *pvl* was not detected despite several attempts using PCR and DNA microarray profiling. Only one isolate per patient was investigated unless AR and PFGE typing indicated the presence of a second strain from a particular patient. Where possible, patient demographics and clinical data were collected from isolate submission forms, telephone follow-ups and follow-up questionnaires. Isolates were defined as clusters if they were recovered from members of the one family/household, or within a hospital or both. Within each cluster, isolates were recovered between three months and two years apart. Each isolate within a cluster was recovered from a different person or environmental source.

3.2.2 Confirmation of isolates as *S. aureus*, methicillin susceptibility testing and detection of the *lukF/S-PV* genes

On receipt by the NMRSARL all *S. aureus* isolates were inoculated onto Protect beads (Technical Service Consultants Limited, Heywood, United Kingdom) and stored at -70°C prior to subsequent investigation. Isolates were confirmed as *S. aureus* as described in Chapter 2, section 2.3. The detection of the *lukF/S-PV* genes was performed by PCR as described previously (Rossney *et al.*, 2007) or, for isolates recovered in the final quarter of 2011 and in 2012, using an in-house real-time PCR assay designed to detect the *mecA*, *nuc* and *pvl* genes. The identification of isolates as *S. aureus*, the presence or absence of

TABLE 3.1 Numbers of *pvl*-positive MRSA and MSSA isolates identified each year between 2002 and 2012 by the Irish National MRSA Reference Laboratory

Year	No. of MRSA isolates			No. of MSSA isolates		
	Identified by NMRSARL	Investigated for <i>pvl</i> ¹	Confirmed as <i>pvl</i> positive (%) ²	Identified by NMRSARL	Investigated for <i>pvl</i> ¹	Confirmed as <i>pvl</i> positive (%) ²
2002	497	8	1 (0.2) ³	1	0	1 (100)
2003	599	17	3 (0.5) ³	0	0	0 (0)
2004	724	134	11 (1.5) ³	15	14	3 (20)
2005	827	112	9 (1.1) ³	43	30	5 (11.6)
2006	869	110	12 (1.4)	41	31	5 (12.2)
2007	782	120	17 (2.2)	42	29	6 (14.3)
2008	747	179	37 (5.0)	58	53	7 (12.1)
2009	605	187	32 (5.3)	44	35	5 (11.4)
2010	596	160	28 (4.7)	77	61	5 (6.5)
2011	456	190	40 (8.8)	81	61	2 (2.5)
2012	579	218	41 (7.1)	102	83	6 (5.9)
Total	7281	1435	231 (3.2)	504	397	45 (8.9)

¹An isolate was selected for *pvl* investigation if it was from a suspected *pvl*-associated infection or, for MRSA only, if the isolate exhibited an antibiogram-resistogram (AR) and pulsed field group (PFG) pattern distinct from that of previously or currently predominant *pvl*-negative healthcare-associated MRSA clones e.g. AR-PFG 06-01, indicative of ST22-MRSA-IV or AR-PFG 13/14-00, indicative of ST8-MRSA-IIA-E +/-SCC_{MI}.

²The value shown in parenthesis indicates the percentage of *pvl*-positive MRSA or MSSA identified among the total number of MRSA or MSSA investigated by the National MRSA Reference Laboratory (NMRSARL) each year during the study period.

³The MRSA isolates recovered between 2002 and 2005 have been described previously (Rossney *et al.*, 2007). One MRSA isolate (E1760) from that study was excluded from the present study because *pvl* was not detected despite several attempts using PCR and DNA microarray profiling.

mecA and the presence of the *lukF/S-PV* genes were also confirmed in all isolates using DNA microarray profiling (Chapter 2, section 2.4.4).

3.2.3 Phenotypic and genotypic characterisation of *pvl*-positive *S. aureus* isolates

3.2.3.1 Phenotypic and genotypic characterisation

All 283 *pvl*-positive *S. aureus* isolates underwent antimicrobial susceptibility testing, *spa* typing (Chapter 2, section 2.3) and DNA microarray profiling (Chapter 2, section 2.4.4). The 24 previously described *pvl*-positive MRSA isolates recovered between 2002 and 2005 included in the study previously underwent antimicrobial susceptibility, MLST and SCC*mec* typing and toxin gene profiling for a limited number of toxin genes (Rossney *et al.*, 2007).

3.2.3.2 MLST

Three of the 18 ST772-MRSA-V isolates investigated underwent MLST as part of the present study as described previously (Enright *et al.*, 2000; Shore *et al.*, 2008). This was performed to confirm them as belonging to this previously unseen genotype in Ireland. This involves PCR and sequencing of seven housekeeping genes (Table 3.2). Amplimers were sequenced commercially by Geneservice Ltd. (Source Bioscience) and resulting sequences were analysed using the BioNumerics software package (Applied Math, Belgium). Sequence types are assigned to isolates by comparing the allele sequences of the seven housekeeping genes to ST alleles on the MLST website (<http://saureus.mlst.net>). Isolates were assigned to CCs if six of the seven alleles were identical to the allelic profile of at least one other ST within a specific CC. For all cases other than the three ST772-MRSA-V isolates which underwent MLST, CCs and STs were assigned using the DNA microarray (Chapter 2, section 2.4.4).

3.2.3.3. Antimicrobial susceptibility testing and PCR detection of antimicrobial resistance genes

The susceptibility of each isolate to a panel of 23 antimicrobial agents was determined by disk diffusion as described in Chapter 2, section 2.3.3. Isolates were further investigated by PCR to confirm the presence or absence of resistance genes detected in these isolates using the DNA microarray if they (i) exhibited phenotypic resistance to particular antimicrobial agents for which associated resistance genes were not detected by the DNA

microarray, or (ii) harboured resistance genes but partial or none of the associated resistance phenotype was not detected. These investigations included PCRs to detect *ileS2*, *aphA3*, *aacA-aphD*, *fusB*, *tet(K)*, *tet(M)*, *aadD*, *qacA*, *qacC*, *msr(A)*, *dfpSI*, *lnu(A)*, *mph(C)* and *blaZ*. Primers and PCR conditions used are shown in Table 2.3, Chapter 2.

Table 3.2 The seven housekeeping genes involved in Multilocus Sequence Typing of *S. aureus* and the primers required (Enright *et al.*, 2000)

Gene	Gene product	Forward Primer (5' -3')	Reverse Primer (5' -3')
<i>arc</i>	Carbamate kinase	TTGATTCCACCAGCGCGTATTGTC	AGGTATCTGTCTTCAATCAGCGG
<i>aroE</i>	Shikimate dehydrogenase	ATCGGAAATCCTATTTCACATTC	GGTGTGTGATTAAATAACGATATC
<i>glp</i>	Glycerol kinase	CTAGGAACTGTCAATCTTAATCC	TGGTAAAATCGCATGTGCCAATTC
<i>gmk</i>	Guanylate kinase	ATCGTTTATCGGGGACCATC	TCATTAACTACAACGTAATCGTA
<i>pta</i>	Phosphate acetyltransferase	GTTAAAATCGTATTACCTGAAGG	GACCCCTTTTGTGAAAAGCTTAA
<i>tpi</i>	Triosephosphate isomerase	TCGTTTCATTCTGAACGTCGTGAA	TTTGCACTTCTAACAATTGTAC
<i>yqiL</i>	Acetyl coenzyme A acetyltransferase	CAGCATACAGGACACCTATTGGC	CGTTGAGGAATCGATACTGTGAAC

3.3 Results

3.3.1 Prevalence of *pvl*-positive MRSA and MSSA

A total of 276 *pvl*-positive *S. aureus* isolates were identified by the NMRSARL between 2002 and 2012. This included 231 *pvl*-positive MRSA and 45 *pvl*-positive MSSA representing 3.2% and 8.9% of all MRSA and MSSA, respectively, submitted to the NMRSARL between 2002 and 2012 (Table 3.1). Overall, the prevalence of *pvl*-positive MRSA among all MRSA submitted to the NMRSARL increased significantly during the study period (p -value = 0.00) from 0.2% in 2002 (1/497) to 7.1% (41/579) in 2012, with 2002 and 2011 recording the lowest and highest prevalence rates, respectively (Table 3.1). For *pvl*-positive MSSA, the prevalence rate among all MSSA submitted to the NMRSARL decreased from 20% in 2004 (3/15) to 5.9% (6/102) in 2012 and the overall numbers of MSSA investigated for *pvl* increased during this time (Table 3.1).

3.3.2 Genotyping

The *pvl*-positive MRSA ($n = 231$) and MSSA ($n = 45$) isolates were assigned to 14 and six MLST (CCs), respectively (Table 3.3). Both MRSA and MSSA were assigned to four of these CCs, namely CC1, CC22, CC30 and CC88 (Table 3.3). Among the MRSA, the isolates were assigned to either SCC mec types IV (78.4%, 181/231) or V (21.6%, 50/231) and 17 genotypes (CC/ST-SCC mec types) were identified with CC/ST8-MRSA-IV predominating (31.2%, 72/231), followed by CC/ST30-MRSA-IV (19.5%, 45/231), CC1/ST772-MRSA-V (13.4%, 31/231), CC/ST80-MRSA-IV (12.6%, 29/231), CC/ST22-MRSA-IV (8.7%, 20/231), ST59/952-MRSA-V (4.3%, 10/231), CC/ST93-MRSA-IV (2.6%, 6/231) (Table 3.2). The remaining 10 MRSA genotypes were each represented by less than four isolates only including CC1-MRSA-V (4/231), CC1-MRSA-IV (1/231), CC5-MRSA-IV (2/231), CC5-MRSA-V (1/231), CC8-MRSA-V (1/231), CC45-MRSA-V (1/231), CC/ST59-MRSA-IV (1/231), CC88-MRSA-IV (3/231), CC/ST154-MRSA-IV (1/231) and CC/ST152-MRSA-V (2/231).

Among the *pvl*-positive MSSA, CC30 was the dominant clone with 40% (18/45) of isolates being assigned to this genotype (Table 3.2). CC22-MSSA accounted for 22.2% (10/45) of MSSA isolates while CC121-MSSA, CC1-MSSA and CC88-MSSA accounted for 20% (9/45), 8.9% (4/45) and 6.7% (3/45) of isolates, respectively (Table 3.2).

A total of 97.5% of *pvl*-positive *S. aureus* isolates (269/276) harboured immune evasion complex (IEC) genes and were assigned to IEC types A-G or to a recently described novel type consisting of *sea* and *scn* (Table 3.2) (vanWamel *et al.*, 2006; Prabhakara *et al.*, 2013). IEC type B (*sak*, *chp* and *scn*) was the most common IEC type identified (59.8%, 165/276) and was found in isolates belonging to nine different genotypes including the majority of isolates in the two most prevalent MRSA (CC8/ST8-MRSA-IV and CC30/ST30-MRSA-IV) and MSSA (CC30-MSSA and CC22-MSSA) genotypes (Table 3.2). IEC type D (*sea*, *sak* and *scn*) was only found in CC1 and CC6 isolates and the novel IEC type (*sea* and *scn*) was unique to ST772-MRSA-V isolates (Table 3.2). IEC type E (*sak* and *scn*) was predominantly associated with CC80/ST80-MRSA-IV, ST152-MRSA-V and CC121-MSSA isolates and IEC type C (*chp* and *scn*) and F (*sep*, *sak*, *chp* and *scn*) were only found in ST59/952-MRSA-V and CC88-MSSA isolates, respectively (Table 3.2). IEC type G was found in a single CC5-MRSA-IV isolate (Table 3.2).

All four *agr* types were identified but types I and III predominated (42.8%, 118/276 and 41.7%, 115/276, respectively) (Table 3.2). Isolates within each CC carried the same *agr* type except for CC1, where the ST772-MRSA-V isolates harbored *agr* II while other CC1 isolates had *agr* III. Clonal complex 5 isolates were the only other isolates to carry *agr* II. Type IV *agr* was only identified in CC121-MSSA (Table 3.2).

Both capsule types 5 and 8 were identified with approximately half of the isolates being assigned to each capsule type (Type 5, 52.2%, 144/276; Type 8, 47.8%, 132/276) (Table 3.2).

3.3.3 Temporal changes in the predominant clonal types of *pvl*-positive MRSA

Figure 3.1 shows the numbers of *pvl*-positive MRSA isolates assigned to each genotype each year between 2002 and 2012. Eleven of the genotypes identified between 2006 and 2012 were not identified between 2002 and 2005, including ST93-MRSA-IV, CC/ST59-MRSA-IV/V and ST772-MRSA-V. The latter was identified for the first time in 2009 where it accounted for just 3.1% (1/32) of isolates but this increased to 28.6% (8/28) in 2010 and it was the predominant genotype in 2011 accounting for 40% (16/40) of *pvl*-positive MRSA isolates. However, the prevalence of this clone declined in 2012 where it was the third most common genotype accounting for 14.6% (6/41) of isolates (Fig. 3.1).

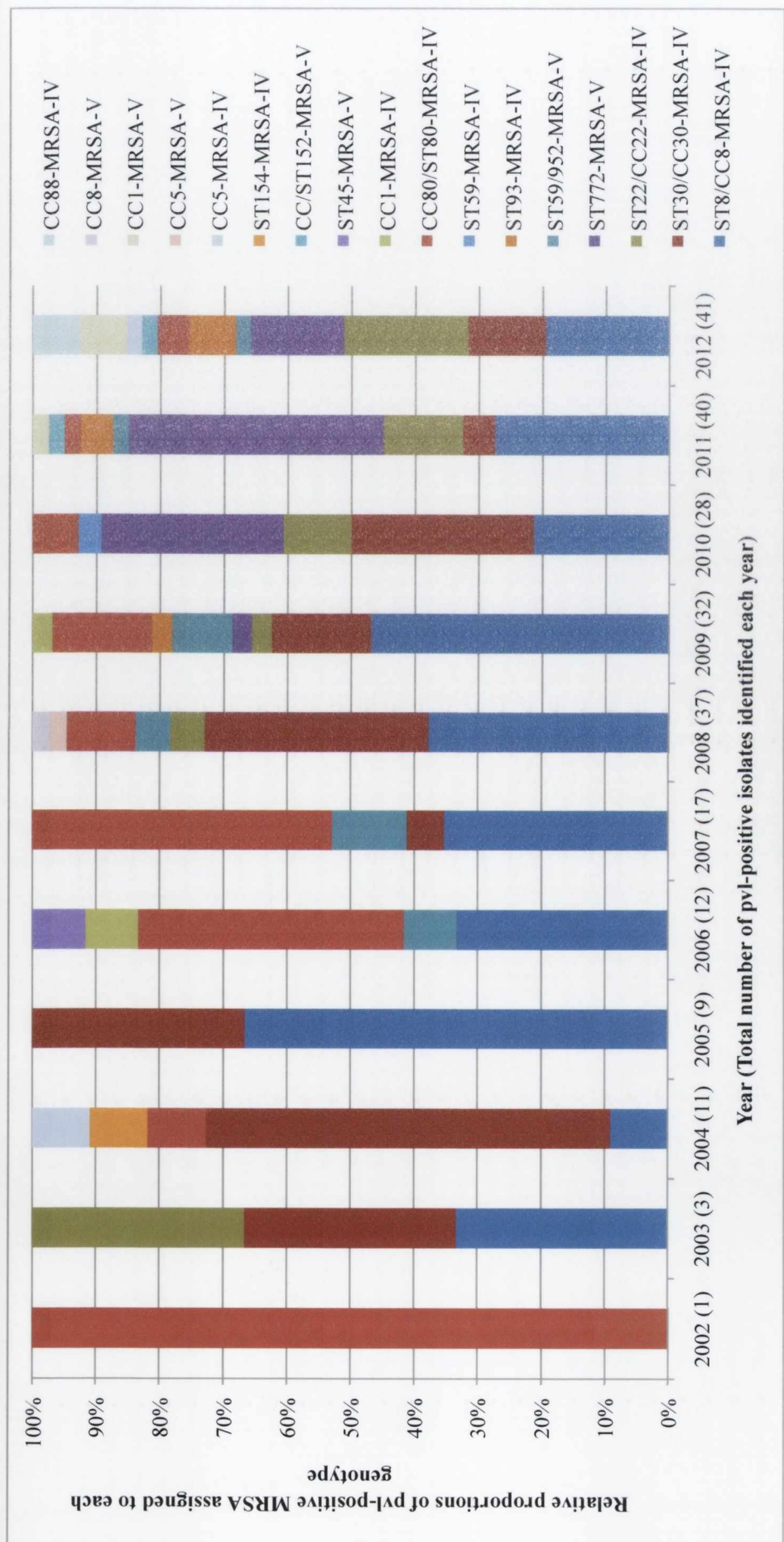


Figure 3.1 The relative proportions of the 231 *pvl*-positive MRSA isolates identified by the Irish National MRSA Reference Laboratory between 2002 and 2012 assigned to each genotype each year between during the study period. Numbers in parenthesis (*n*) indicate the number of *pvl*-positive MRSA isolates identified each year.

Table 3.3 Phenotypic and genotypic typing data for *pvl*-positive MRSA (*n* = 231) and MSSA (*n* = 45) isolates identified by the Irish National MRSA Reference Laboratory between 2002 and 2012

CC	Genotype (n) ¹	spa types (n) ²	agr type ¹	Capsule type ¹	IEC type (n) ^{1,2,3}	Antibiotic resistance profiles	Virulence	
	MRSA							
						Resistance phenotype (% indicated when not 100%) ⁴	Resistance genotype (% indicated when not 100%)	Virulence genes (% indicated when not 100%)
1	CC1-MRSA-IV (2)	t128 (1), t8968 (1)	III	8	D (1), B (1)	Ap, Cd , Te (50)	blaZ, sdrM, tet(K) (50)	sea (50), sec&sel (50), sek&seq (50), seh
	ST772-MRSA-V (31)	t008 (1), t657 (29), t345 (1)	II	5	Novel: sen & sea	Ap (96.8), Ak, Cd (96.8), Cp (96.8), Er, Gn (96.8), Kn, Nm, Tb (96.8), Tp	blaZ, sdrM, msr(A), mph(C), aacA-aphD, aphA3&sat, fosB	sea, sec&sel, egc
	CC1-MRSA-V (4)	t127	III	8	D	Ap, Fd, Gn, Kn, Nm, Tb, Te	blaZ, sdrM, aacA-aphD, aphA3 & sat, tet(K) (25), tet(M) (25), fusC	sea, sek&seq, seh
5	CC5-MRSA-V (1)	t127 (2), t177 (1), t12303 (1)	III	8	D	Ap, Cd (75), Fd, Kn (25), Nm (25)	blaZ, sdrM, aphA3 & sat (25), ileS2 (50), fusC, qacA(50)	sea, sek&seq, seh
	CC5-MRSA-IV (2)	t311	II	5	A	Ap, Cd, Te, Tp	blaZ, sdrM, tet(K), dfpSI, fosB	sea, edinA, egc
6		t304	I	8	D	Ap	blaZ, sdrM, fosB	sea, edinA (50), egc, sed/fjr (50)
	CC6-MSSA (1)						blaZ, vgaA, fosB, sdrM	None detected
8	CC/ST8-MRSA-IV (72)	t008 (53), t051 (4), t068 (2), t121 (3), t4229 (1), t304 (1), t024 (1), t681 (1), t4306 (1), t11157 (1), t596 (1), t1610 (1), t1635 (1)	I	5	B (71), neg (1)	Ak (19.4), Ap, Cd (20.8) (1), C1 (1.4), Cp (41.7), Er (74.9), Gn (1.4), Kn (77.8), Ln (2.8), Mc (8.3), Mp (1.4), Nm (77.8), Pma (1.4), Tb (1.4), Te, (9.7) Tp (4.2), Ln (1.4)	blaZ (90.3), sdrM, tet(K) (8.3), lnu(A) (2.7), msr(A) (80.6), mph(C) (83.3), aacA-aphD (1.3), aphA3 & sat (76.4), fosB, merA & merB (6.9), qacC (4.2), ileS2 (1.3), cfr & fexA(1.3), dfpSI (2.8)	sek&seq (94.4), sed/fjr (4.2), ACME (86.1)

CC	Genotype (n) ¹		spa types (n) ²	agr type ¹	Capsule type ¹	IEC type (n) ^{1,2,3}	Antibiotic resistance profiles			Virulence
	MRSA	MSSA					Resistance phenotype (% indicated when not 100%) ⁴	Resistance genotype (% indicated when not 100%)	Virulence genes (% indicated when not 100%)	
			t2689 (1)							
	CC8-MRSA-V (1)		t008	I	5	B	Ap	<i>sdrM</i> , <i>fosB</i>	<i>sek&seq</i> , ACME	
22	CC/ST22-MRSA-IV (20)		t852 (13), t2480 (1), t3107 (1), t4463 (1), t5422 (2), t005 (2)	I	5	B (19), neg (1)	Ak (15), Ap, Cd (30), Cp (80), Er (70), Gn (15), Kn (90), Nm (20), Tb (90), Tp (70)	<i>blaZ</i> , <i>aacA-aphD</i> (90), <i>dfpSI</i> (70), <i>ermC</i> (65), <i>aadD</i> (75)	<i>egc</i> , <i>sec&sel</i> (5)	
30	CC/ST30-MRSA-IV (45)	CC22- MSSA (10)	t005 (7), t891 (2), t1869 (1)	I	5	B	Ak (10), Ap, Cd (60), Fd (10), Gn (80), Kn, Tb (90), Tp (90)	<i>blaZ</i> , <i>aacA-aphD</i> , <i>dfpSI</i> (90)	<i>egc</i>	
			t019 (25), t012 (12), t021 (1), t3800 (2), t122 (1), t275 (1), t318 (1), t9904 (1), t6653 (1)	III	8	B (42), A (2), neg (1)	Ap, Cd (75.6), Cp (6.7), Fd (31.1), Te (2.2), Tp (2.2)	<i>blaZ</i> , <i>sdrM</i> , <i>tetK</i> (2.2), <i>fosB</i> , <i>dfpSI</i> (2.2), <i>fusC</i> (31.1)	<i>sea</i> (4.4), <i>tst</i> (31.1), <i>egc</i>	
		CC30- MSSA (18)	t021 (8), t318 (4), t990 (1), t1741 (1), t3502 (1), t1055 (1), t11156 (1), t433 (1)	III	8	A (2), B (14), neg (3)	Ap (77.8), Cd (83.3), Cp, Er (11.1), Kn (5.6), Nm (5.6), Te (16.7), Tp (16.7)	<i>blaZ</i> (77.8), <i>mphC</i> (6.7), <i>sdrM</i> , <i>tetK</i> (11.1), <i>fosB</i> , <i>dfpSI</i> (5.6), <i>msrA</i> (5.6), <i>mphC</i> (5.6), <i>aphA3&sar</i> (5.6)	<i>sea</i> (11.1), <i>sec&sel</i> (5.6), <i>sek&seq</i> (5.6), <i>tst</i> (11.1), <i>egc</i> (88.9)	
45	CC45-MRSA-V (1)		t620	I	8	B	Ap, Te	<i>blaZ</i> , <i>sdrM</i> , <i>tetK</i>		
59	ST59/952-MRSA-V (10)		t437	I	8	C	Ap, Cd (20), Cl (90), Er (90), Kn (90), Ln (90), Nm (90), St (80), Te (60)	<i>blaZ</i> (80), <i>sdrM</i> , <i>tetK</i> (70), <i>aphA3 & sar</i> (90), <i>ermB</i> (90), <i>cat-pC223</i> (90)	<i>seb</i> , <i>sek&seq</i>	

CC	Genotype (n) ¹		spa types (n) ²	agr type ¹	Capsule type ¹	IEC type (n) ^{1,2,3}	Antibiotic resistance profiles			Virulence
	MRSA	MSSA					Resistance phenotype (% indicated when not 100%) ⁴	Resistance genotype (% indicated when not 100%)	Virulence genes (% indicated when not 100%)	
	ST59-MRSA-IV (1)		t437	I	8	A	Ap, Er, Kn, Ln, Nm, St	<i>blaZ</i> , <i>sdrM</i> , <i>aphA3</i> & <i>sat</i> , <i>erm(B)</i>	<i>sea</i> , <i>seb</i> , <i>sek</i> & <i>seq</i>	
78 80	CC80/ST80-MRSA-IV (29)		t042 (2), t044 (21) t376 (5) t131 (1)	III	8	E	Ak (3.4), Ap, Cd (86.2), Cl (3.4), Cp (3.4), Er (41.4), Fd (75.9), Kn, Nm, Te (79.3), Tp (3.4)	<i>blaZ</i> (79.3), <i>sdrM</i> , <i>dfpSI</i> (3.7), <i>tet(K)</i> (72.4), <i>aphA3</i> & <i>sat</i> , <i>fusB</i> (72.4), <i>erm(C)</i> (41.4), <i>tet(K)</i> (72.4), <i>cat-pC221</i> (3.5), <i>dfpSI</i> (3.5)	<i>etD</i> & <i>edimB</i>	
88	CC88-MRSA-IV (3)		t692 (2), t11016 (1)	III	8	E (2), F (1)	Ap, Cd (66.7), Cp (33.3), Er (33.3), Kn (66.7), Mc (66.7), Tb (66.7), Tp (66.7)	<i>blaZ</i> , <i>aadD</i> (66.7), <i>aphA3</i> (66.7), <i>ermC</i> (33.3), <i>dfpSI</i> (66.7), <i>tetK</i> , <i>sdrM</i>	None detected	
93	ST93-MRSA-IV (6)	CC88-MSSA (3)	t186 (2) t448 (1)	III	8	F	Ap, Cd (66.7), Er (33.3), Te (66.7), Tp (33.3)	<i>blaZ</i> , <i>sdrM</i> , <i>tet(K)</i> (66.7), <i>erm(C)</i> (33.3), <i>dfpSI</i> (33.3)	<i>sek</i> & <i>seq</i> (33.3)	
121		CC121-MSSA (9)	t3949 (1) t1819 (1) t202 (4)	III	8	B	Ap, Cd (50), Cp (16.7), Er (33.3), Gn (16.7), Kn (16.7), Ln (16.7), Nm (16.7), Tb (16.7), Tp (16.7)	<i>blaZ</i> , <i>sdrM</i> , <i>msrA</i> (16.7), <i>mphC</i> (16.7), <i>qacC</i> (16.7)	<i>entCM14</i> (50)	
152	ST152-MRSA-V (2)		t159 (5) t435 (2) t4198 (1), t2304 (1)	IV	8	E	Ap, Cd (22.2) (1), Cl (11.1), Er (44.4), Ln (11.1), St (11.1), Te (33.3), Tp (33.3)	<i>blaZ</i> , <i>sdrM</i> , <i>tet(K)</i> (33.3), <i>fosB</i> (88.9), <i>erm(C)</i> (44.4), <i>cat-pC221</i> (11.1), <i>dfpSI</i> (22.2)	<i>seb</i> (66.7), <i>egc</i> , <i>entCM14</i> (77.8)	
154	ST154-MRSA-IV (1)		t355	I	5	E	Ap, Cd, Gn, Kn, Tb	<i>blaZ</i> , <i>sdrM</i> , <i>aacA-aphD</i>	<i>etD</i> & <i>edimB</i>	
			t667	III	8	Neg	Ap, Cd (50), Cp, Sp, Te	<i>blaZ</i> , <i>sdrM</i> , <i>tet(M)</i> , <i>cat-pMC524</i>	None detected	

¹The StaphyType DNA microarray Kit (Alere Technologies) was used for assigning isolates to a multilocus sequence type (MLST) sequence type (ST) and/or a clonal complex (CC), a staphylococcal cassette chromosome *mec* (SCC*mec*) type (for MRSA only), and accessory gene regulator (*agr*) and capsule and immune evasion complex (IEC) types. Forty-three MRSA isolates previously underwent MLST and SCC*mec* typing including 18 ST772-MRSA-V, two ST22-MRSA-IV, 11 ST30-MRSA-IV, eight ST8-MRSA-IV, two ST80-MRSA-IV, the one ST154-MRSA-IV and the one ST5-MRSA-IV isolates (Rossney *et al.*, 2007; Brennan *et al.*, 2012).

²The number of isolates (*n*) represented by each *spa* type or IEC type are indicated in parenthesis only when more than one *spa* or IEC type was identified within a genotype.

³Immune evasion complex (IEC) types were defined as described previously : A= *sea*, *sak*, *chp* and *scn*; B = *sak*, *chp* and *scn*; C = *chp* and *scn*; D = *sea*, *sak* and *scn*; E = *sak* and *scn*; F = *sep*, *sak*, *chp* and *scn*; novel = novel IEC type consisting of *sak* and *sea* (ref, Van wamel, 772 phage ref); neg (negative) = no IEC genes detected.

⁴The susceptibility of each isolate to a panel of 23 antimicrobial agents was determined by disk diffusion as described previously (Rossney *et al.*, 2006). The antimicrobial agents tested were Amikacin (Ak), Ampicillin (Ap), Cadmium acetate (Cd), ciprofloxacin (Cp), erythromycin (Er), ethidium bromide (Eb), fusidic acid (Fd), gentamicin (Gn), kanamycin (Kn), lincomycin (Ln), mercuric chloride (Mc), mupirocin (Mp), neomycin (Nm), phenyl mercuric acetate (Pma), rifampicin, spectinomycin (Sp), streptomycin (St), sulphonamide, tetracycline (Te), tobramycin (Tb), trimethoprim (Tp) and vancomycin.

Abbreviations: neg, negative

The two most predominant *pvl*-positive MRSA clones identified during the study period, CC/ST8-MRSA-IV and CC/ST30-MRSA-IV, were first detected in 2003 (one isolate each, accounting for 25% of isolates in 2003). The CC/ST30-MRSA-IV clone predominated and was at its most prevalent in 2004 when it accounted for 70% of *pvl*-positive MRSA isolates (7/10). The prevalence of CC/ST30-MRSA-IV declined in 2005 to 33.3% (3/9) and this clone was not detected in 2006, accounted for just 5.9% of isolates (1/17) in 2007, but its prevalence increased significantly in 2008 to 35.1% (13/37) where it predominated together with CC/ST8-MRSA-IV. Following a decline in 2009 to 15.6% (5/32), the prevalence of this clone again increased in 2010 to 28.6% (8/28) when it predominated together with ST772-MRSA-V but declined again in 2011 to just 5% (2/40) and increased in 2012 to 12.2% (5/41) (Fig. 3.1).

The CC/ST8-MRSA-IV clone predominated and was at its most prevalent in 2005 where it accounted for 66.7% of isolates (6/9). Despite a decrease to 33.3% (4/12) in 2006, a rise in the prevalence of this clone was noted between this and 2009 to 46.9% (15/32). The CC/ST8-MRSA-IV clone again became the predominant clone (together with CC/ST30-MRSA-IV) in 2008 (37.8%, 14/37) and in 2009 (46.9%, 15/32). The predominance of this clone decreased to 21.4% in 2010 (6/28) but increased again in 2011 (27.5%, 11/40), where it was the second most common clone identified following ST772-MRSA-V. Subsequently, there was a slight decline in 2012 to 19.5% (8/41) in 2012, but together with ST22-MRSA-IV, it was the most common clone identified in 2012 (Fig. 3.1).

Apart from 2002 when just one *pvl*-positive MRSA isolate was identified and was assigned to CC80/ST80-MRSA-IV, the highest prevalence of this clone was in 2007 when it accounted for 47.1% of isolates (8/17 isolates). The prevalence of this clone subsequently declined in 2008 to 10.8% and despite an increase in 2009 (15.6%), it subsequently declined to 4.9% in 2012 (2/41 isolates) (Fig. 3.1).

Prior to 2008, just one *pvl*-positive ST22-MRSA-IV isolate was detected (2003). Despite low numbers of isolates, an increase in the prevalence of *pvl*-positive ST22-MRSA-IV was noted between 2009 (3.1%, 1/32) and 2012 (19.5%, 8/41) (Fig. 3.1). The first ST59/952-MRSA-V isolates were detected in 2006 and a small number of isolates of this clone were subsequently detected each year apart from 2010 (between one and four isolates each year) (Fig. 3.2). The highest prevalence of this clone was in 2009 (12.5%,

4/32). Only six ST93-MRSA-V isolates were identified, one in 2009, two in 2011 and three in 2012. Two clones were only detected in the latter years of the study although the numbers of isolates were low; this included CC1-MRSA-V (one isolate in 2011 and three in 2012) and CC88-MRSA-IV (three isolates only, all detected in 2012). All other clones were represented by one or two isolates only (Fig. 3.1).

3.3.4 Characteristics of *pvl*-positive *S. aureus* isolates

The virulence and resistance gene profiles of isolates identified within each genotype of *pvl*-positive MRSA and MSSA are shown in Table 3.2 and Figs. 3.2 and 3.3 and the main characteristics of isolates within lineages i.e. CCs, represented by more than one isolate, are described below.

CC1. The majority of CC1/ST772-MRSA-V isolates exhibited *spa* type t657 (93.5%, 29/31) and all exhibited resistance to multiple antimicrobial agents including ciprofloxacin, trimethoprim, erythromycin and aminoglycosides, the latter two encoded by *msr(A)/mph(C)* and *aacA-aphD/aphA3*, respectively. The enterotoxins *sec* & *sel* and *egc* as well as the novel IEC type consisting of *scn* and *sea* were identified in all ST772-MRSA-V isolates.

The other CC1 genotypes identified (CC1-MRSA-IV, CC1-MRSA-V and CC1-MSSA) exhibited different *spa*, *agr*, capsule and IEC types to CC1/ST772-MRSA-V. The CC1-MRSA-V isolate also exhibited resistance to multiple antimicrobial agents and carried multiple resistance genes but apart from aminoglycoside resistance encoded by *aphA3* and *aacA-aphD*, these were different to those detected in CC1/ST772-MRSA-V and included tetracycline resistance encoded by *tet(K)* and *tet(M)* and fusidic acid resistance encoded by *fusC*. The two CC1-MRSA-IV isolates carried less resistance genes with just one isolate carrying *tet(K)*. The CC1-MRSA-IV/V isolates lacked *egc* but various other enterotoxin genes were detected including *sea*, *sec* & *sel*, *sek* & *seq* and *seh*.

Of the four CC1-MSSA isolates identified, two exhibited the same *spa* type, t127, as the CC1-MRSA-V isolate. Multiple resistance genes were detected among these isolates including *aphA3*, *fusC*, *ileS2* and *qacA* but for the latter two phenotypic resistance to mupirocin and quaternary ammonium compounds was not detected. Similar toxin genes to those detected in CC1-MRSA were detected among the CC1-MSSA isolates,



Figure 3.2 The relative proportions of enterotoxin, exfoliative toxin and toxic shock toxin genes and the arginine catabolic mobile element (ACME) detected among each genotype of *pvl*-positive MRSA and MSSA identified by the Irish National MRSA Reference Laboratory between 2002 and 2012. Numbers in parenthesis indicate the number (n) of *pvl*-positive MRSA or MSSA isolates assigned to each genotype.

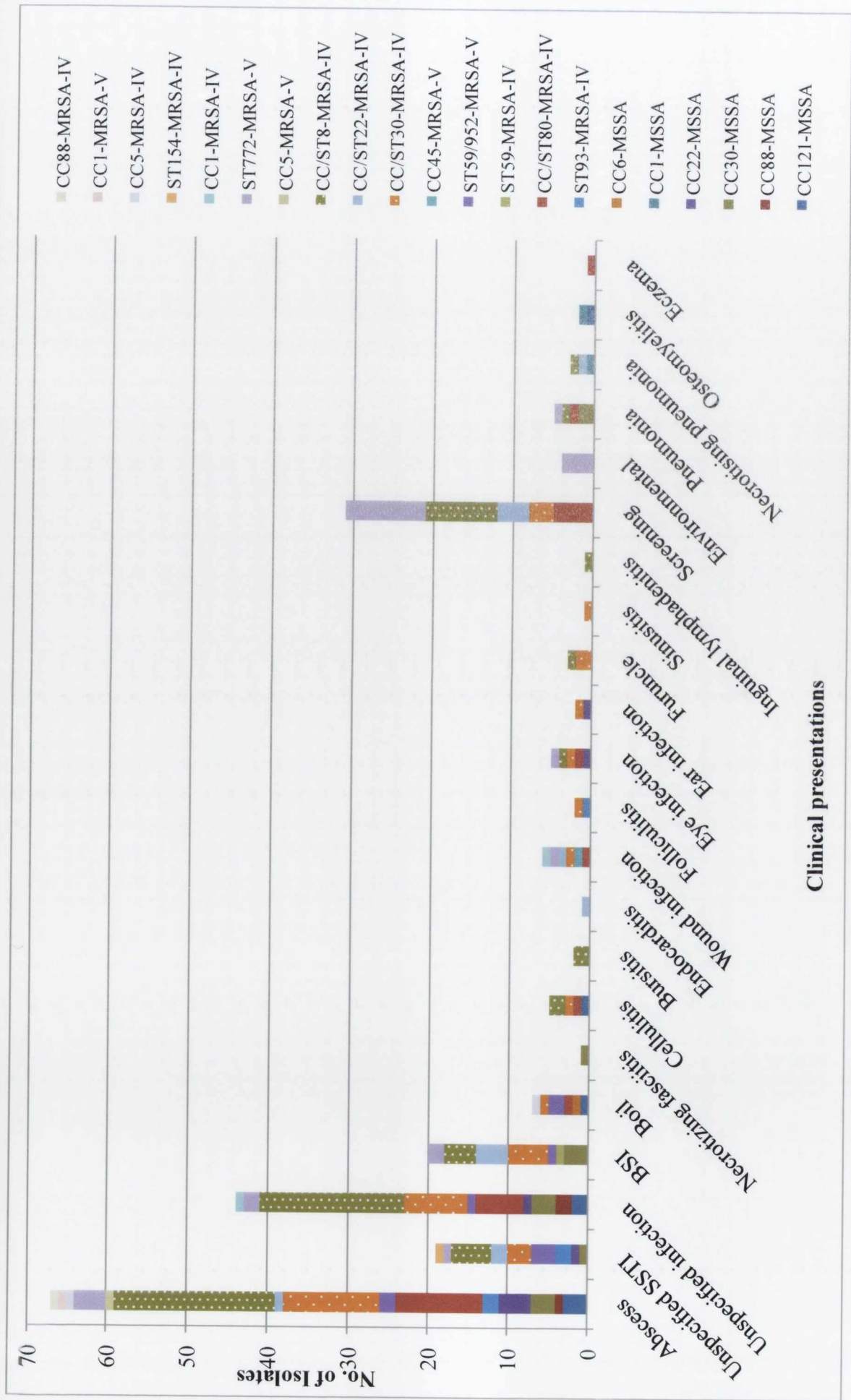


Figure 3.3 Clinical details for each genotype identified among the *pvl*-positive MRSA and MSSA isolates investigated. Clinical details were available for 197 MRSA and 35 MSSA isolates. Abbreviations: SSTI, skin and soft tissue infection; BSI, bloodstream infection.

namely *sea*, *sek* & *seq* and *seh*. In fact, *seh* was unique to CC1 and was detected in all isolates except those belonging to ST772.

CC5. Of the three CC5 MRSA isolates identified, two harboured SCC*mec* IV and the other SCC*mec* V, but they all exhibited the same *agr* and capsule type. One of the CC5-MRSA-IV harboured *spa* type t214 and IEC type G, while the remaining CC5-MRSA-IV and CC5-MRSA-V isolates carried the same *spa* and IEC type. Only the CC5-MRSA-V isolate carried *dfrS1* and *tet(K)* and exhibited resistance to trimethoprim and tetracycline, respectively, and all isolates carried *sea* and *egc*.

CC8. Although 14 *spa* types were identified among the CC/ST8-MRSA-IV isolates, t008 predominated (73.6%, 53/72). The majority of CC/ST8-MRSA-IV isolates exhibited resistance to kanamycin and neomycin encoded by *aphA3* and erythromycin encoded by *msr(A)* and almost half of the isolates were resistant to ciprofloxacin. Just under 10% of CC/ST8-MRSA-IV isolates were tetracycline resistant and carried *tet(K)*. One isolate carried *cfr* and *fexA* and exhibited chloramphenicol and linezolid resistance (Shore *et al.*, 2010). The majority of isolates carried the enterotoxin genes *sek* and *seq* and ACME and, although they were less common, *sed*, *sej* and *ser* were also identified.

The one remaining CC8-MRSA t008 isolate harbored SCC*mec* V and did not exhibit resistance to multiple antimicrobial agents or harbor multiple resistance genes but *sek* & *seq* and ACME were detected. ACME was only identified in one non CC/ST8-MRSA isolate (CC45-MRSA-V).

CC22. The *spa* types t852 and t005 predominated among the CC22 MRSA (65%, 13/20) and MSSA (70%, 7/10), respectively. Only one *spa* type, t005, was common to both CC22 MRSA and MSSA but only two t005 MRSA isolates were identified. Among the CC22-MRSA-IV isolates resistance to aminoglycosides encoded by *aacA-aphD* and *aadD*, trimethoprim encoded by *dfrS1*, erythromycin encoded by *erm(C)* and ciprofloxacin, were common. No ciprofloxacin resistant CC22 MSSA isolates were identified but they all exhibited aminoglycoside resistance encoded by *aacA-aphD*, the majority were resistant to trimethoprim and carried *dfrS1* and one isolate exhibited resistance to fusidic acid which was probably due to mutations in *fusA* as neither *fusB* or *fusC* were detected. However, not all CC22 isolates harboring *aacA-aphD* and *aadD* exhibited resistance to all of the appropriate aminoglycoside antimicrobial agents. All

CC22 isolates carried *egc* and other toxin genes *sec* & *sel*, were detected in only a single CC22-MRSA-IV isolate.

CC30. The majority of CC/ST30-MRSA-IV isolates were assigned to *spa* type t019 (55.6%, 25/45) or t012 (26.7%, 12/45). Less than a third of the isolates were resistant to fusidic acid encoded by *fusC* and resistance to tetracycline and trimethoprim encoded by *tet(K)* and *dfpS1*, respectively, was detected in just one isolate each. All CC/ST30-MRSA-IV isolates carried *egc*, 31.1% (14/45) carried the toxic shock toxin gene *tst* with just two isolates harboring *sea*.

Among the CC30 MSSA *spa* types t021 (44.4%, 8/18) and t318 (22.2%, 4/18) predominated. These two *spa* types (t021 and t318) were the only common *spa* types detected among CC30 MRSA and MSSA but were only detected in one CC30-MRSA isolate each. While no fusidic acid resistance phenotype or genes were detected among the CC30-MSSA, resistance to tetracycline, trimethoprim and erythromycin encoded by *tet(K)*, *dfpS1* and *mph(C)*, respectively, was identified in up to three isolates each. CC30-MSSA isolates carried the greatest range of toxin genes i.e. *sek* & *seq*, *egc*, *sea*, *sec* & *sel* and *tst*, but apart from *egc*, which was detected in the majority of CC30 MSSA, each of these were found in one or two CC30-MSSA isolates only. Overall, *tst* was unique to CC30 isolates.

CC59. All ST59/952-MRSA-V isolates exhibited a single *spa* type, t437, and the majority of isolates exhibited resistance to multiple antimicrobial agents and carried multiple resistance genes with erythromycin and lincomycin resistance encoded by *erm(B)*, kanamycin and neomycin resistance encoded by *aphA3* and chloramphenicol resistance encoded by *cat-pC223*. Tetracycline resistance encoded by *tet(K)* was also common among these isolates. All ST59/952-MRSA-V isolates carried the enterotoxin genes *seb* and *sek* & *seq*.

The one ST59-MRSA-IV isolate identified carried less resistance genes but *aphA3* and *erm(B)* encoding resistance to aminoglycosides and erythromycin, respectively, were detected. Similar to the ST59/952-MRSA-V isolates, the ST59-MRSA-IV isolate carried *seb* and *sek* & *seq* but *sea* was also detected.

CC80. The majority of CC/ST80-MRSA-IV isolates exhibited *spa* type t044 (72.4%, 21/29). All isolates exhibited resistance to kanamycin and neomycin encoded by

aphA3. Resistance to tetracycline, fusidic acid and erythromycin encoded by *tet(K)*, *fusB* and *erm(C)*, respectively, were also common. However, for a small number of isolates, *tet(K)* and *fusB* were identified but the appropriate resistance phenotype was not detected. Chloramphenicol and trimethoprim resistance encoded by *cat-pC221* and *dfrS1*, respectively, as well as ciprofloxacin resistance were detected in just one isolate each. All CC/ST80-MRSA-IV isolates harbored the exfoliative toxin gene *etD* and the epidermolytic toxin *edinB*, which were identified in just one other isolate (ST152-MRSA-V).

CC88. Six CC88 isolates, three MRSA and three MSSA were identified and were assigned to two *spa* types each. The CC88-MSSA and one CC88-MRSA isolate were the only isolates found to harbor IEC type F (*sep*, *sak*, *chp* and *scn*). Two MSSA isolates exhibited tetracycline resistance and carried *tet(K)* with just one isolate each exhibiting resistance to erythromycin and trimethoprim encoded by *erm(C)* and *dfrS1*, respectively. The MRSA isolates all carried *tet(K)* but did not exhibit phenotypic tetracycline resistance, and the majority exhibited resistance to aminoglycosides and mercuric chloride, with a single isolate exhibiting resistance to ciprofloxacin and erythromycin. The enterotoxin genes *sek* & *seq* were detected in one CC88-MSSA isolate and no other virulence genes were detected among either the CC88-MRSA or MSSA

CC93. The six CC/ST93-MRSA-IV isolates exhibited three different *spa* types with t202 predominating (66.7%, 4/6). Erythromycin resistance encoded by *msr(A)* and *mph(C)* was detected in just two isolates. The *qacC* gene was also detected in one isolate but the isolate did not exhibit resistance to ethidium bromide. The enterotoxin gene *CM14* was the only toxin gene detected and was found in 50% of CC93-MRSA-IV isolates.

CC121. All CC121 isolates identified were MSSA and the majority exhibited *spa* type t159 (55.6%, 5/9). Only CC121-MSSA isolates exhibited *agr* type IV. Just under half of the isolates exhibited erythromycin resistance encoded by *erm(C)* and tetracycline, trimethoprim and chloramphenicol resistance encoded by *tet(K)*, *dfrS1* and *cat-pC221*, respectively, were also detected among CC121 MSSA. All CC121-MSSA isolates harbored *egc* and *CM14* was found in over three-quarters of isolates and two-thirds also carried *seb*.

3.3.5 Multiresistant *pvl*-positive MRSA

Multiresistance was identified among MRSA isolates only and was defined as phenotypic resistance to three or more classes of commonly used antimicrobial agents tested including fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin/kanamycin/neomycin/tobramycin), macrolides/lincosamides (erythromycin-/lincomycin), tetracyclines, fusidic acid and mupirocin (Ellington *et al.*, 2010). Using this criterion, 42.4% (98/231) of *pvl*-positive MRSA isolates were multiresistant (Table 3.4 and Fig. 3.5). These multiresistant *pvl*-positive MRSA isolates were assigned to six genotypes, with the majority of isolates belonging to CC/ST8-MRSA-IV (28.6%, 28/98), ST772-MRSA-V (25.5%, 25/98), and CC/ST80-MRSA-IV (23.5%, 23/98) with a small number of multiresistant isolates also belonging to CC/ST22-MRSA-IV (12.2%, 12/98), ST59/952-MRSA-V (6.1%, 6/98) and CC1-MRSA-V (4.1%, 4/98) (Fig. 3.5). An increase in the prevalence of multiresistant *pvl*-positive MRSA was observed between 2004 (10%, 1/10) and 2007 (59%, 10/17) ($p < 0.02$) and despite a decline in 2008 (24.3%, 9/37), this again increased between 2008 and 2011 to 65% (26/40) ($p < .001$) with a slight decrease in 2012 to 36.6% (15/41) (Fig. 3.1). Overall the highest prevalence of multiresistance among *pvl*-positive MRSA isolates was observed in 2011 and this was predominantly associated with isolates within ST772-MRSA-V (61.5%, 16/26) and to a lesser extent, CC/ST8-MRSA-IV (19.2%, 5/26) CC/ST22-MRSA-IV (11.5%, 3/26), ST59-MRSA-V (3.8%, 1/26) and CC1-MRSA-V (3.8%, 1/26).

3.3.6 Patient demographics

Of the 276 isolates investigated, 261 (94.6%) were from patients, 11 from healthcare staff (4%) and four (1.4%) from environmental sources. Information pertaining to whether the *S. aureus* isolates were from patients based in the community or in hospitals were available for 181/276 isolates, 68.5% (124/181) of whom were based in the community.

3.3.6.1 Sex and age

Gender data was available for patients from whom 229 isolates were recovered, of which, 49.8% were from males (114/229) and 50.2% (115/229) were from females (Table 3.4). There was no significant difference between the genders of patients associated with *pvl*-positive MRSA and MSSA. The ages of patients from whom *pvl*-positive *S. aureus* was

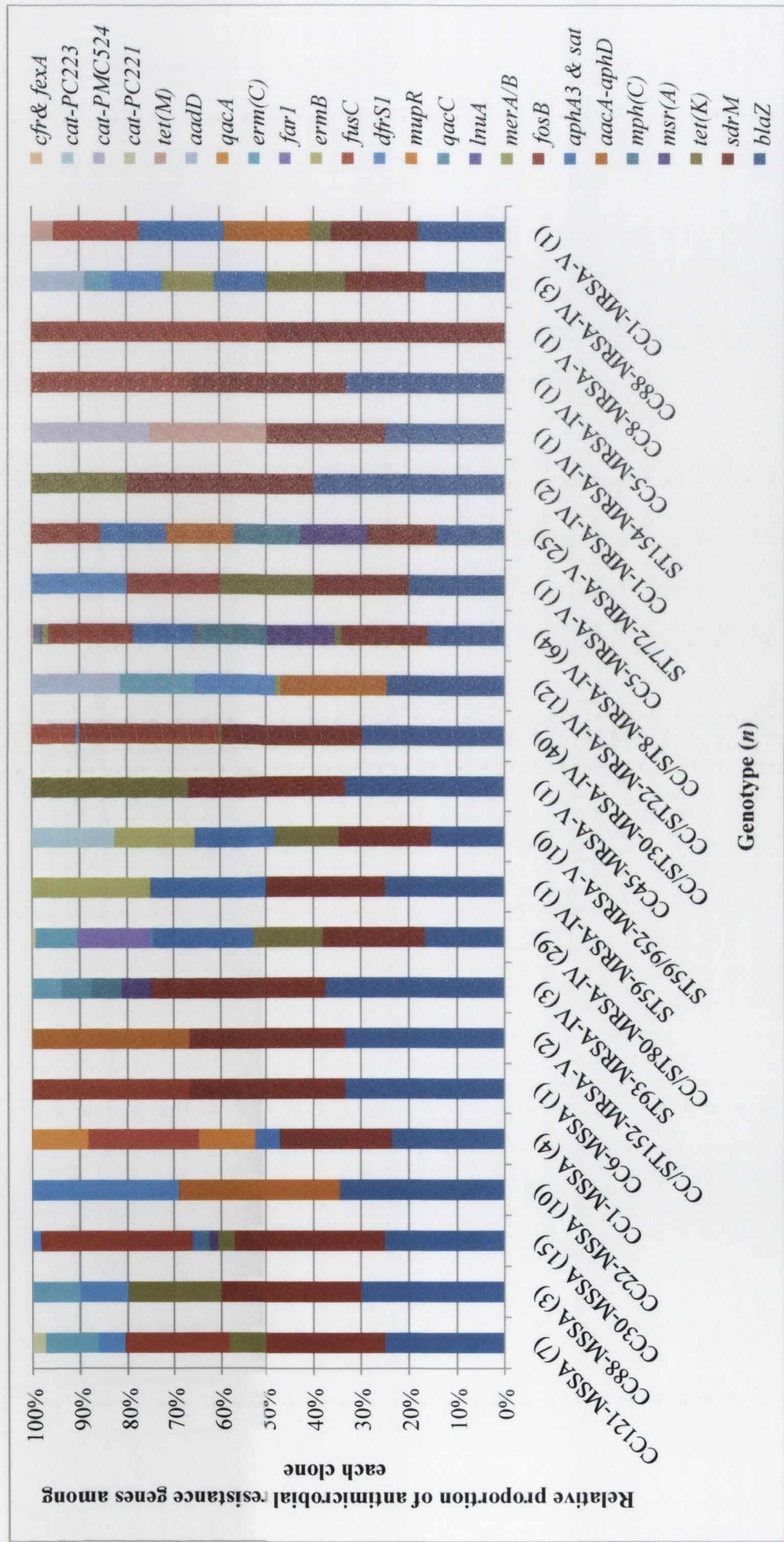


Figure 3.4 The relative proportions of antimicrobial resistance genes among each genotype of *pvl*-positive MRSA and MSSA identified by the Irish National MRSA Reference Laboratory between 2002 and 2012. Numbers in parenthesis indicate the number (n) of *pvl*-positive MRSA or MSSA isolates assigned to each genotype.

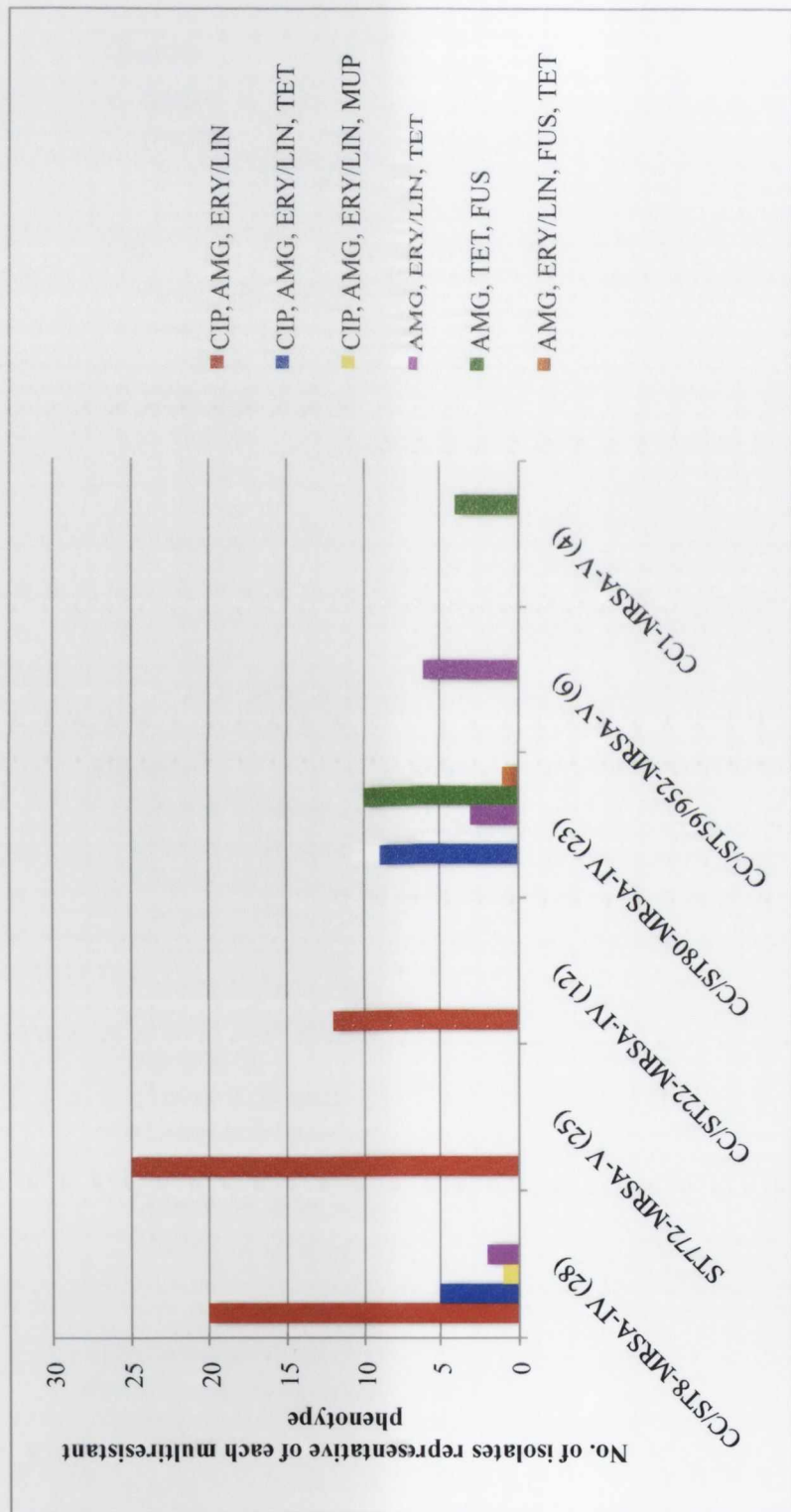


Figure 3.5 Multiresistant phenotypes detected among *pv*-positive MRSA. Ninety-eight of the 231 *pv*-positive MRSA isolates exhibited multiresistance (42.4%) i.e. exhibited resistance to three or more classes of clinically used antimicrobial agents tested including fluoroquinolones (ciprofloxacin, CIP), aminoglycosides (AMG), erythromycin/lincomycin (ERY/LIN), tetracycline (TET), mupirocin (MUP) and fusidic acid (FUS). Numbers (*n*) in parenthesis indicate the number of multiresistant isolates within each genotype.

Table 3.4 Patient demographics associated with *pvl*-positive MRSA and MSSA identified by the Irish National MRSA Reference Laboratory between 2002 and 2012

Category	No. of isolates	Genotype ¹
Gender		
Male	114	n/a
Female	115	n/a
Data not available	37	n/a
Age		
Age range (yrs)		
0-9	41	n/a
10-19	20	n/a
20-29	48	n/a
30-39	53	n/a
40-49	27	n/a
50-59	14	n/a
60-69	11	n/a
70-79	9	n/a
80-89	6	n/a
90-99	3	n/a
Data not available	34	n/a
Clusters²		
Cluster 1 (Household)	4	CC/ST30-MRSA-IV
Cluster 2 (Household)	3	CC/ST30-MRSA-IV
Cluster 3 (Household)	4	CC/ST8-MRSA-IV
Cluster 4 (Household)	3	CC/ST8-MRSA-IV
Cluster 5 (Household)	4	CC80-MRSA-IV
Cluster 6 (Hospital)	6	ST772-MRSA-V
Cluster 7 (Hospital & household)	11	ST772-MRSA-V
Cluster 8 (Household)	2	ST772-MRSA-V
Cluster 9 (Household)	3	CC1-MRSA-V
Cluster 10 (Household)	2	ST93-MRSA-IV
Cluster 11 (Hospital)	2	ST22-MRSA-IV
Cluster 12 (Hospital)	3	CC30-MSSA
International travel to or country/region of origin		
USA	1	CC1-MRSA-IV
	3	ST8-MRSA-IV
Africa	1	ST8-MRSA-IV
	1	CC5-MRSA-IV
	2	ST30-MRSA-IV
	2	CC121-MSSA
	1	CC5-MRSA-V

Continued Overleaf

Category	No. of isolates	Genotype ¹
Asia-the Middle East	1	ST80-MRSA-IV
Asia-India	9	ST772-MRSA-V
Asia-the Far East	1	ST80-MRSA-IV
	1	ST154-MRSA-IV
	3	ST30-MRSA-IV
	1	CC121-MSSA
	1	ST59-MRSA-V
	1	ST8-MRSA-IV
	1	CC88-MRSA-IV
Australia	1	CC22-MRSA-IV
	1	ST93-MRSA-IV
New Zealand	1	ST8-MRSA-IV
South America-Brazil	2	ST8-MRSA-IV
Brazil	1	CC30-MRSA-IV
Europe-The Czech Republic	1	CC1-MRSA-IV
Spain	1	ST59/952-MRSA-V
Undefined (outside of Ireland)	2	ST59/952-MRSA-V
	1	ST22-MRSA-IV
	1	CC22-MSSA
	1	CC30-MSSA

¹CC, MLST clonal complex; ST, sequence type.

²Isolates were defined as clusters if they were recovered from members of the one family/household, or within a hospital or both. Within each cluster isolates were recovered between three months and two years apart. Each isolate within a cluster was recovered from a different person or environmental source. Abbreviations: n/a, not applicable.

recovered ranged from <1 month to 98 years and the median age was 30 years (age data was available for 242 patients). Sixty seven percent (162/242) of isolates were from patients who were <40 years of age (Table 3.4).

3.3.6.2 Clinical presentations

Clinical data was available for 232 isolates (197 MRSA and 35 MSSA) (Fig. 3.4). The most common infections were SSTIs (52.2%, 121/232) including unspecified SSTIs, abscesses, boils, furuncles, bursitis, folliculitis, sinusitis, eye and ear infections, inguinal lymphadenitis and wound infections. SSTIs were associated with isolates from all except three genotypes, CC1-MSSA ($n = 4$), ST152-MRSA-V ($n = 2$) and ST59-MRSA-IV ($n = 1$). More serious manifestations were also identified including BSIs (8.6%, 20/232) belonging to ST59-MRSA-IV, ST59/952-MRSA-V, ST30-MRSA-IV, ST22-MRSA-IV, ST8-MRSA-IV, CC1-MRSA-IV, ST772-MRSA-V and CC30-MSSA), pneumonia (2.6%, 6/232 isolates belonging to CC30-MSSA, CC121-MSSA, ST772-MRSA-V, CC/ST8-MRSA-IV and CC/ST80-MRSA-IV), osteomyelitis (0.9%, 2/232 isolates; CC121-MSSA and CC1-MSSA), necrotising pneumonia (1.3%, 3/232 isolates; CC/ST8-MRSA-IV and CC1 MSSA), necrotising fasciitis (0.4%, 1/232, CC30-MSSA) and endocarditis (0.4%, 1/232 isolates, CC22/ST22-MRSA-IV). Thirty-one isolates were recovered during screening of patients (nose, throat and/or perineum sites) during hospital outbreaks or from persons with close contact with patients with *pvl*-positive *S. aureus*.

3.3.6.3 International travel or country of origin outside of Ireland

Forty-three individuals from whom *pvl*-positive isolates were recovered were known to have recently travelled internationally or had a country of origin outside of Ireland (Table 3.4). Recent travel ranged from two weeks to one year prior to the recovery of the *pvl*-positive *S. aureus* isolate from the patient, but for the majority of patients the time period since travel was not defined. The genotypes most commonly associated with international travel or country of origin outside of Ireland were ST772-MRSA-V (nine isolates), ST8-MRSA-IV (eight isolates), and ST30-MRSA-IV (six isolates) (Table 3.4). The ST8-MRSA-IV and ST30-MRSA-IV isolates were identified from patients with links to multiple regions worldwide, while the ST772-MRSA-V isolates were exclusively associated with travel to and/or from India (Table 3.4).

Overall the most common travel destination or region of origin for patients with *pvl*-positive *S. aureus* was Asia (including India, the Far East and the Middle East), which was represented by 19 isolates belonging to seven MRSA genotypes and one MSSA genotype and ST772-MRSA-V, ST30-MRSA-IV and ST80-MRSA-IV were each represented by more than one patient with links to this region (Table 3.4). The next most common regions were Africa (seven isolates consisting of five genotypes, two each belonging to CC5-MRSA, ST30-MRSA-IV and CC121-MSSA) and the USA (3/4 isolates CC8/ST8-MRSA-IV). One ST93-MRSA-IV and one ST59/952-MRSA-V isolate were recovered from patients who had travelled to Australia and the Far East, respectively, (Table 3.4).

3.3.6.4 Isolate clusters

One cluster was identified among *pvl*-positive MSSA and seven among *pvl*-positive MRSA, either from two or more members of one family/household or within a hospital, or both (Table 3.4). Within each cluster isolates were recovered between three months and two years apart and isolates were represented by a single genotype with indistinguishable *spa* and DNA microarray profiles in each case. CC1/ST772-MRSA-V was associated with three clusters, CC/ST8-MRSA-IV and CC/ST30-MRSA-IV were associated with two clusters each with CC80-MRSA-IV, CC1-MRSA-V, ST93-MRSA-IV, ST22-MRSA-IV and CC30-MSSA accounting for one cluster each. ST772-MRSA-V accounted for over a third of all cluster-associated isolates identified (40.4%, 19/47) and included both of the hospital clusters (in two neonatal ICUs in separate maternity hospitals), one of which also had an associated household cluster and is described below in more detail (Table 3.5 and section 3.3.7).

3.3.7 ST772 cluster investigation

Over a seven-month period between October 2010 and May 2011 seven babies in a neonatal ICU (NICU) in an Irish maternity hospital were found to be colonised by ST772-MRSA-V. All parents and staff members screened for MRSA after the first isolates were recovered in October 2010 were found to be negative for MRSA. During a second episode of staff screening in March 2011, 148 staff were screened and five were identified as MRSA carriers, two of whom were found to carry an indistinguishable strain to the ST772-MRSA-V recovered from the neonates (Table 3.5). Both of these two staff members (one was from India (yielded isolate M11/0092) and one from Ireland (yielded

TABLE 3.5 Epidemiological, clinical, phenotypic and genotypic characteristics of the cluster of hospital-associated and community-associated ST772-MRSA-V isolates recovered in Ireland between 2009 and 2011

Hospital	Isolate no.	MRSA- positive site	Age	Ethnicity	Relevant clinical information	Location acquired/source	<i>spa</i> type	Antimicrobial resistance pattern ¹	Antimicrobial resistance ²	Virulence associated genes ²	<i>agr</i> / capsule type ²
H1	M10/0338	Nasal, umbilicus, perineum	7 days	Irish	Carriage cluster in NICU ³	In patient	t657	Ak ⁴ , Ap, Cd ⁵ , blaZ, msr(A), Cp ⁶ , Er, Gn, mph(C), Kn, Nm, Tb, aacA-aphD,	lukF-PV & lukS-PV,		II/5
H1	M10/0342	Nasal	15 days	Irish	Carriage cluster in NICU ³	In patient	t657	aphA3 & sat, fosB, sdrM	sea, sec & sel, egc (seg, sei, sem, sen, seo & seu),		
H1	M10/0349	Nasal, umbilicus	20 days	Irish	Carriage cluster in NICU ³	In patient	t657		scn, bbp, cflA, clfB, cna, ebh,		
H1	M11/0082	Nasal, umbilicus	3 days	Irish	Carriage cluster in NICU ³	In patient	t657		ebpS, eno, fib, fnbA, fnbB, map,		
H1	M11/0085	Nasal, umbilicus	24 days	Irish	Carriage cluster in NICU ³	In patient	t657		icaA, icaC, icaD, sdrC, sdrD, vwb, sasG		
H1	M11/0107	Umbilicus	9 days	Irish	Carriage cluster in NICU ³	In patient	t657				
H1	M11/0120	Nasal, umbilicus	9 days	Irish	Carriage cluster in NICU ³	In patient	t657				
H1	M11/0092	Nasal, axilla, groin	29 years	Indian	Staff member, probable index case	Staff working in NICU	t657				

Continued Overleaf

Hospital	Isolate no.	MRSA-positive site	Age	Ethnicity	Relevant clinical information	Location acquired/source	<i>spa</i> type	Antimicrobial resistance ¹ pattern ¹	Antimicrobial resistance ²	Virulence associated genes ²	<i>agr</i> /capsule type ²
H1	M11/0097	Unknown	35 years	Irish	Staff member	Staff working in NICU	t657				
H1	M11/0167	Nasal	16 months	Indian	Child of the probable index case	Family contact of colonized NICU staff member	t657				
H1	M11/0093	Environment	N/A ⁷	N/A ⁷	Horizontal surface in NICU ³	N/A ⁷	t657				

¹ Antimicrobial resistance was determined by antibiogram-resistogram typing against a panel of 23 antimicrobial agents including Ak, amikacin; Ap, ampicillin; Cd, cadmium acetate; chloramphenicol; Cp, ciprofloxacin; Er, erythromycin; ethidium bromide; fusidic acid; Gn, gentamicin; Kn, kanamycin; lincomycin; mercuric chloride; mupirocin; Nm, neomycin; phenyl mercuric acetate; rifampin; spectinomycin; streptomycin; sulfonamide; tetracycline; Tb, tobramycin; Tp, trimethoprim; vancomycin.

² Antimicrobial resistance genes, virulence-associated genes, *agr* type and capsule type determined using the StaphyType DNA microarray kit (Alere, Germany).

³ NICU, neonatal intensive care unit.

⁴ 14/18 isolates exhibited resistance to amikacin while four isolates (M11/0035, M10/0045, M10/0131 and M10/0203) were susceptible.

⁵ 15/18 isolates exhibited resistance to cadmium acetate and two (M11/0035 and M10/0033) were susceptible.

⁶ 17/18 isolates exhibited resistance to ciprofloxacin and one isolate (M11/0035) was susceptible.

⁷ N/A, not applicable.

isolate M11/0097) had worked in the NICU at the time when the babies yielded positive MRSA cultures. Epidemiological investigation indicated that one of the staff members (yielded isolate M11/0092) was the probable index case due to her intimate involvement in the care for all babies who were colonised with ST772-MRSA-V. The staff member had worked in the NICU for a number of years prior to the recovery of the first ST772-MRSA-V isolate, however she had recently been hospitalised during a visit to India, where ST772-MRSA-V predominates. Prior to decolonisation treatment of this staff member, ST772-MRSA-V was recovered from her nose, groin and axilla but she had no symptoms of infection. Following decolonisation treatment, undertaken by the occupational health department, screening swabs were negative for MRSA on two occasions. However, the ST772-MRSA-V strain was recovered from a groin swab from a third set of screening swabs. Family contacts of the probable index case were screened and a nasal swab from her young child (yielded isolate M11/0167), who had been born in India, was also found to carry this strain.

The decolonisation protocol applied to staff members consisted of a five-day course of 2% mupirocin nasal ointment applied to the inner surface of each nostril three times daily and use of an octenidine-based shower gel/shampoo. Babies over 36-week gestation were also bathed in octenidine and treated with mupirocin. One of the staff members (yielded isolate M11/0097) colonised with the ST772-MRSA-V strain was successfully decolonised. The probable index case proved difficult to decolonise and as such underwent the decolonisation protocol twice. Following decolonisation a screening throat swab collected from her child yielded the same ST772-MRSA-V strain. Treatment for this throat carriage was not possible using a mouth wash due to the age of the child.

3.3.7.1 Environmental screening

Following environmental screening of approximately 30 areas including the main reception, staff kitchen and clinical areas, MRSA was recovered from one area of the NICU, a horizontal surface in the area of one baby associated with the ST772-MRSA-V cluster (isolate M11/0093, Table 3.5). The environmental cleaning regimen, which included the cleaning of all equipment in the patient zone area, (e.g. monitors, suction machines, intravenous pumps, canopy of the incubator etc.) with detergent wipes and which was carried out daily within the unit, was increased to twice daily.

3.3.7.2 Characterisation of MRSA isolates

The 11 MRSA isolates from patients, staff members, the child of the probable index case and the environmental isolate from the NICU exhibited a similar multi-antibiotic resistance phenotype (Table 3.5). All MRSA isolates exhibited *spa* type t657 and DNA microarray analysis assigned the isolates to CC1 and ST573/772 with SCCmec type V. All isolates harboured multiple antibiotic resistance genes including those encoding resistance to macrolides (*msr(A)* and *mph(C)*) and aminoglycosides (*aacA-aphD* and *aphA3*) (Table 3.4). All isolates were positive for the PVL genes *lukF-PV* and *lukS-PV*, the enterotoxin genes *sea*, *sec* and *sel*, the enterotoxin gene cluster (*egc*) along with the IEC gene *scn* but the β -toxin gene *hly* was not detected, either in its intact or disrupted form (Table 3.5). Genes encoding adhesion and biofilm factors were also detected (Table 3.5).

3.3.7.3 Additional ST772-MRSA-V isolates

Comparison of the antimicrobial susceptibility pattern of the ST772-MRSA-V isolates recovered in the NICU to all MRSA isolates submitted to the NMRSARL until 2011 identified seven additional ST772-MRSA-V that were indistinguishable from those involved in the NICU (Table 3.5). Two of these isolates were from patients in two separate Irish hospitals and five from community sources. Subsequently, over the following two years a further 13 ST772-MRSA-V isolates were identified (Table 3.5).

3.4 Discussion

The results of this study highlight several novel findings in relation to *pvl*-positive MRSA, including an increase in the prevalence and diversity of *pvl*-positive MRSA submitted to the NMRSARL between 2002 and 2012, and several temporal shifts in the predominant clonal types. Overall, 3.2% (231/7281) of MRSA isolates submitted to NMRSARL during the ten-year study period were *pvl*-positive but a significant increase, from 0.2% to 7.1%, was observed between 2002 and 2012 (Fig 3.1). While these findings may reflect a true increase in the prevalence of *pvl*-positive MRSA in Ireland over the last decade, enhanced clinical and laboratory awareness of *pvl* and consequently an increase in the numbers of *pvl*-positive MRSA isolates referred to the NMRSARL for investigation, probably contributed to the higher rate. However, a relatively low but increasing prevalence of *pvl*-positive MRSA has also been reported from some other European countries during the last decade, including Austria and Germany (Witte *et al.*, 2007; Berkthold *et al.*, 2012).

3.4.1 The changing epidemiology of *pvl*-positive MRSA in Ireland

The polyclonal *pvl*-positive MRSA population structure that has been identified in Ireland as part of the present study and also in other European countries (Ellington *et al.*, 2010; Berkthold *et al.*, 2012; Rolo *et al.*, 2012; Brauner *et al.*, 2013) is in stark contrast to the USA and Australia where single epidemic *pvl*-positive clones predominate, specifically ST8-MRSA-IV/USA300 and ST93-MRSA-IV, respectively (Tenover *et al.*, 2006; Coombs *et al.*, 2012). Many reasons have been proposed for the differences in prevalence rates and genetic diversity of *pvl*-positive MRSA in the USA compared to Europe including environmental, host, social, economic and cultural differences (Witte, 2009; Rolo *et al.*, 2012). However, direct evidence for these is somewhat lacking and many of the risk factors identified for *pvl*-positive MRSA/CA-MRSA identified in the USA may also apply to various communities in Europe such as poverty, overcrowding, homelessness, participation in contact sports, military service and intravenous drug use (Charlebois *et al.*, 2002; CDC, 2003; CDC, 2004). In the present study, while such specific parameters were not investigated, nine familial/household outbreaks of *pvl*-positive MRSA were identified. Furthermore, links between several *pvl*-positive *S. aureus* isolates and patients with recent foreign travel or ethnic origin outside of Ireland were

also identified, highlighting the continuing role of strain importation on the influx of a variety of *pvl*-positive MRSA strains into Ireland.

The results of this study also suggest that the importation of *pvl*-positive MRSA strains is more significant than the local emergence of *pvl*-positive MRSA from *pvl*-positive MSSA. While four of the six MSSA lineages were also identified among MRSA, just 3.5% (8/231) of MRSA isolates exhibited the same *spa* type as MSSA isolates including, t127 (CC1), t005 (CC22) and t318 (CC30). Due to the greater abundance of these *spa* types among *pvl*-positive MSSA (identified in 31.1% of MSSA isolates) it is reasonable to speculate that this small number of *pvl*-positive MRSA may have evolved from the *pvl*-positive MSSA by the acquisition of *SCCmec* rather than loss of *SCCmec* by MRSA, although both alternatives are possible.

While the prevalence of the different *pvl*-positive MRSA clones identified in the present study together with the precise temporal shifts of the predominant clones are unique to Ireland, both similarities and differences were noted in comparison with polyclonal *pvl*-positive MRSA populations observed in other European countries. For example, a decline in the incidence of the *pvl*-positive European Clone ST80-MRSA-IV, a clone recently shown, using whole-genome sequencing, to have been originally been introduced into Europe from West Africa (Stegger *et al.*, 2014), has been noted recently across Europe in association with an increase in ST8-MRSA-IV/USA300 (Rolo *et al.*, 2012).

In the present study, an increase in the prevalence of ST8-MRSA-IV/USA300 was observed between 2006 and 2009, and it predominated in 2008 and 2009, decreased in 2010 but it was the second most common clone in 2011, surpassed only by ST772-MRSA-V. ST8-MRSA-IV was again the most common *pvl*-positive clone in 2012, although there was an overall decline in its prevalence. The emergence of ST772-MRSA-V as the dominant *pvl*-positive MRSA clone in 2011 in Ireland reflects a similar situation in the UK where ST772-MRSA-V was the predominant multiresistant *pvl*-positive MRSA clone between 2005 and 2008 (Ellington *et al.*, 2012). In 2012 the prevalence of ST772 declined in Ireland however it was still the third most prevalent MRSA clone only slightly behind ST22 and ST8. The predominance of ST772-MRSA-V and ST8-MRSA-IV/USA300 among *pvl*-positive MRSA in Ireland is of concern for several reasons. Firstly, both clones appear to be highly transmissible with a propensity to spread

worldwide and both have been shown to spread within hospitals, and to have an ability to displace hospital strains (D'Souza *et al.*, 2010; O'Hara *et al.*, 2012). This is reflected in the findings of the present study where ST772-MRSA-V was found in association with two separate hospital clusters and two familial clusters and ST8-MRSA-IV/USA300 with two family clusters. Both of these strains have frequently been imported into Ireland, most often in association with foreign travel or country of origin outside of Ireland. Secondly, in addition to carriage of *pvl*, genetic characteristics that may enhance their virulence or ability to spread were detected among isolates of these clones including ACME and the enterotoxin genes *sek* & *seq* in ST8-MRSA-IV/USA300 and a *sea* and *pvl*-encoding bacteriophage (Prabhakara *et al.*, 2013) as well as multiple additional enterotoxin genes in ST772-MRSA-V (Table 3.3). Lastly, isolates belonging to both clones can exhibit multiresistance (Ellington *et al.*, 2010). In fact, 80.6% of ST772-MRSA-V isolates and 38.5% of ST8-MRSA-IV/USA300 in the present study were multiresistant.

While the overall numbers of *pvl*-positive ST22-MRSA-IV isolates investigated in this study were low, a 3.5-fold increase in its prevalence was noted between 2008 and 2012, and in 2012 it was the most common clone along with ST8-MRSA-IV (Fig. 3.1). Worryingly, *pvl*-positive ST22-MRSA-IV has been associated with both hospital and community outbreaks elsewhere (Harris *et al.*, 2012; Pinto *et al.*, 2012; Yamamoto *et al.*, 2012) and it now predominates together with ST772-MRSA-V in hospitals in India (D'Souza *et al.*, 2010). Although *pvl*-negative ST22-MRSA-IV is currently the predominant clone in Irish hospitals and has been since 2002, in the present study *pvl*-positive ST22-MRSA-IV were found to be genetically distinct from these in terms of *spa* type, the former predominantly being t032 (Shore *et al.*, 2010) and the latter t852, indicating the independent evolution of these strains.

The South-West Pacific clone (CC/ST30-MRSA-IV) was the second most common *pvl*-positive MRSA clone identified accounting for 19.5% of all *pvl*-positive MRSA isolates during the study period and predominating several times (Fig. 3.1). Isolates of this pandemic clone are also common in the UK and have been associated with a hospital outbreak where the probable index case was a member of staff who had recently travelled to the Philippines (Ellington *et al.*, 2009; Ali *et al.*, 2012; Pantelides *et al.*, 2012). Interestingly, in the present study a link between travel to, or ethnic origin in, Asia or Africa was identified for several CC/ST30-MRSA-IV isolates emphasising the

role that travel potentially plays in the spread of this clone. Isolates of this clone were also associated with two familial outbreaks, indicating its propensity to spread. The prevalence of CC/ST30-MRSA-IV declined dramatically in the present study between 2004 and 2006 from 70% to 0% and again between 2010 and 2011, from 28.6% to 5% with a slight increase again in 2012 to 12% (Fig. 3.1). A decline in the prevalence of this once predominant *pvl*-positive clone among CA-MRSA was also recently reported in New Zealand where it was replaced by *pvl*-negative ST5-MRSA-IV (Williamson *et al.*, 2013). It is now well established that not all CA-MRSA carry *pvl*. In the present study, 70% of *pvl*-positive *S. aureus* isolates for which information was available were from patients based in the community indicating that CA *S. aureus* have emerged as a significant problem in Ireland. However, the true burden of CA *S. aureus* infections in Ireland will only be fully understood when *pvl*-negative as well as *pvl*-positive CA *S. aureus* isolates are investigated systematically together with detailed epidemiological information.

The diversity of *pvl*-positive MRSA clones increased in the second half of the study period with 11/17 MRSA genotypes identified for the first time between 2006 and 2012 including CC/ST59-MRSA-V, ST93-MRSA-IV and ST772-MRSA-V. Links between several isolates of these clones and the regions where they predominate were also noted. Although an increase in the Taiwan clone (CC/ST59-MRSA-V), from 8.3% in 2006 to 12.5% in 2009, was observed, the number of isolates recovered each year remained low throughout (between one and four isolates each year). The incidence of the CC/ST59-MRSA-V also increased in some other European countries in recent years and it is among the predominant CA-MRSA clones in some Northern European countries (Rolo *et al.*, 2012). In contrast, similar to the present study, ST93-MRSA-IV has only been reported sporadically in Europe (Ellington *et al.*, 2010; Rolo *et al.*, 2012) but it is the dominant *pvl*-positive MRSA strain in Australia where it has spread into healthcare facilities (Munckhof *et al.*, 2008).

Despite a decrease in 2008, an overall six-fold increase in the prevalence of multiresistant *pvl*-positive MRSA was identified between 2004 and 2011. Similarly, a 12.3-fold increase in the prevalence of multiresistant *pvl*-positive MRSA was noted in the UK between 2006 and 2008 (Ellington *et al.*, 2010). Both in Ireland and the UK, this was largely due to the emergence and predominance of ST772-MRSA-V and, in Ireland only, the continued prevalence of ST8-MRSA-IV/USA300. However, in the present study, between 2011 and 2012, there was a decline in the prevalence of multiresistant *pvl*-

positive MRSA from 65% to 36.6%. Detailed analysis of *pvl*-positive MRSA isolates recovered during subsequent years is required to determine if this trend has continued to the present day or if multiresistance has again increased. Despite this the prevalence of multiresistant ST22-MRSA-IV isolates increased six-fold between 2009 and 2012; each year over 66% of the *pvl*-positive ST22-MRSA-IV isolates exhibited multiresistance. The increase in multiresistant *pvl*-positive MRSA and the multitude of genes encoding resistance to many clinically used antimicrobial agents identified is alarming as many of them are known to be located on MGEs, indicating their ability to spread. Also, of concern is the high prevalence of ciprofloxacin resistance identified among multiresistant *pvl*-positive MRSA isolates (63.8%). All of these findings highlight how non-multiantibiotic resistance and susceptibility to ciprofloxacin can no longer be considered to be reliable markers for *pvl*-positive MRSA.

The increasing reports of outbreaks of *pvl*-positive MRSA, particularly in NICUs and pediatric units, is alarming and highlights the ability of these strains to spread among vulnerable patient groups in hospitals (Schlebusch *et al.*, 2010; Ali *et al.*, 2012; Harris *et al.*, 2012; Pinto *et al.*, 2012). In the present study two hospital clusters in NICUs in separate maternity hospitals were due to the recently emerged *pvl*-positive multiresistant ST772-MRSA-V clone. In fact, 30% of *pvl*-positive isolates for which information was available were from patients in hospitals, a situation that requires close monitoring so that *pvl*-positive MRSA does not become widespread in Irish hospitals.

3.4.2 Emergence of ST772-MRSA-V in Ireland

The present study describes a ST772-MRSA-V cluster among staff and patients in an Irish NICU and the subsequent retrospective identification of isolates of this strain from patients in other hospitals and in the community in Ireland. Epidemiological data identified that the likely source of the ST772-MRSA-V cluster in the NICU as a staff member from India. Epidemiological and DNA microarray analysis identified multiple subsequent importations of ST772-MRSA into Ireland. The use of *spa* typing in conjunction with the DNA microarray indicated that there was a possible outbreak or a cluster of MRSA within the NICU and this was confirmed by DNA microarray analysis. However the ST772-MRSA-V isolates recovered from the NICU were not associated with infections. All ST772-MRSA-V isolates identified in the NICU exhibited the same multi-antibiotic resistant AR pattern that was distinctly different to that exhibited by the

most frequently occurring MRSA clone in Irish hospitals (ST22-MRSA-IV) (Rossney *et al.*, 2006). DNA microarray analysis assigned the isolates in this cluster as ST772-MRSA-V and identified that they harboured similar virulence and antimicrobial resistance genes to each other and to previously reported ST772-MRSA-V isolates. However, unlike some of the ST772-MRSA-V isolates previously reported by Monecke *et al.* (2011) and Sanchini *et al.* (2011) the isolates in the present study lacked the antimicrobial resistance genes *erm*(C) and *tet*(K) and the enterotoxin genes *sek* and *seq*.

Unlike other ST772-MRSA-V isolates previously reported in the literature (David *et al.*, 2006; Alfroz *et al.*, 2008; Neela *et al.*, 2009; D'Souza *et al.*, 2010; Ellington *et al.*, 2010; Monecke *et al.*, 2011) those recovered within the Irish maternity hospital NICU were not associated with any known clinical diagnosis of infection and were most frequently recovered from screening specimens of patients within the NICU. In contrast the 20 ST772-MRSA-V isolates recovered from patients in other Irish hospitals and from patients in the community during the investigation and for which there was clinical information available were found to be predominantly associated with skin and soft tissue infections with three patients suffering the more serious manifestations of BSI ($n = 2$) and pneumonia ($n = 1$).

The increased spread of CA-MRSA in India has been associated with severe soft tissue infections however there has been an increase in the number of cases associated with bacteraemias affecting neonates (D'Souza *et al.*, 2010). The decreasing prevalence of the HA-MRSA strain ST239-MRSA-III in hospitals in India since 2006 coupled with an increase in prevalence of *pvl*-positive ST22-MRSA-IV and ST772-MRSA-V has led to the suggestion that these strains may be replacing the ST239-MRSA strain in Indian hospitals ((D'Souza *et al.*, 2010). Within Ireland it has been shown that previously predominant strains of MRSA in Irish hospitals have also been replaced at different time periods by different strains (Shore *et al.*, 2005). In 1989 the most frequently occurring strain was ST239-MRSA-III while in 1993 this was replaced by ST8-MRSA-IIA-E & SCC_{MI} (Rossney & Keane, 2002; Shore *et al.*, 2005). The most recent change in the predominant nosocomial MRSA strains in Ireland occurred between 1998 and 2002 where the frequency of ST22-MRSA-IV increased with this strain now accounting for approximately 80% of MRSA bloodstream infections investigated in the NMRSARL each year (NMRSARL 2014). The ST22-MRSA-IV clone could be displaced at some

stage in the future, possibly by ST772-MRSA-V, which, as the situation in India has shown, is capable of displacing previously successful nosocomial MRSA strains.

3.4.3 *pvl*-positive MSSA in Ireland

This study has, for the first time, provided important insights into the molecular epidemiology of *pvl*-positive MSSA in Ireland. Unsurprisingly, the MSSA isolates exhibited greater genetic diversity than the MRSA but the prevalence of *pvl*-positive MSSA decreased during the study period from 20% in 2004 to 2.5% 2011 followed by a slight increase to 5.9% in 2012, with *pvl*-positive MSSA accounting for just 16.3% of all *pvl*-positive isolates identified by the NMRSARL during the study period. In contrast, in the UK the prevalence of *pvl*-positive MSSA increased nine-fold between 2005 and 2010 accounting for 61.5% of all *pvl*-positive *S. aureus* in 2009 (Otokunefor *et al.*, 2012) and in Africa *pvl*-positive MSSA are also common with 57% of MSSA isolates in one study being identified as *pvl*-positive (Breurec *et al.*, 2011). However, it is important to emphasise that MSSA isolates are not routinely referred to the Irish NMRSARL and that the number of MSSA isolates referred each year were low (Table 3.1). Additional studies are required in order to determine the true burden of *pvl*-positive MSSA in Ireland.

Similar to a recent UK study, CC22 and CC30 were the most common *pvl*-positive MSSA clones identified accounting for 62.2% of isolates (Otokunefor *et al.*, 2012). While not reported previously from the UK (Otokunefor *et al.*, 2012), the CC121-MSSA clone that accounted for 20% of *pvl*-positive MSSA isolates in the present study is a pandemic clone (Monecke *et al.*, 2007; Kurt *et al.*, 2013). Interestingly, links to Africa and the Far East were noted for 3/9 CC121 MSSA isolates where this clone has been shown to dominate (Ghasemzadeh-Moghaddam *et al.*, 2011; Kurt *et al.*, 2013). CC88-MSSA accounted for just 6.7% of *pvl*-positive MSSA isolates and have been reported previously from India (Afroz *et al.*, 2008) but isolates of this lineage are more commonly reported as MRSA with SCC*mec* IV, particularly in Africa (Ghebremedhin *et al.*, 2009).

3.4.4 Conclusion

In conclusion, while this study has highlighted the changing molecular epidemiology of *pvl*-positive MRSA and MSSA in Ireland over the last decade it is clear that the actual burden of *pvl*-positive and CA *S. aureus* infections in Ireland is probably higher since this study investigated only *pvl*-positive isolates and only those submitted to the NMRSARL.

There is a need for ongoing and systematic surveillance of *pvl*-positive and CA *S. aureus* infections in communities and hospitals in Ireland together with detailed epidemiological information in order to fully understand the burden of *S. aureus* infections that exists. Infection control practices in hospitals and communities need to be carefully considered to prevent the spread of CA *S. aureus*.

Chapter 4

***pvl*-encoding phage identified among *pvl*-positive *S. aureus* isolates in Ireland**

4.1 Introduction

Staphylococcus aureus, especially MRSA, has been an important causative agent of HCA bacterial infections in humans world-wide for many years. In recent years community-associated *S. aureus* infections have emerged as a significant problem in many countries. The success of *S. aureus* strains in the community has been attributed to its ability to express a wide variety of virulence factors including exotoxins such as haemolysins, enterotoxins, exfoliative toxins, PVL and superantigens such as TST (Otto, 2010; Otto, 2013). The PVL toxin has been linked to SSTIs and the more severe manifestations of necrotising pneumonia and fasciitis. The PVL toxin lyses neutrophils, leukocytes and macrophages by forming pores in the cell membranes. However, its effects are concentration dependent and at sublytic concentrations PVL enhances innate host immunity by triggering inflammasome activation and the release of chemokines (Graves *et al.*, 2012; Perret *et al.*, 2012).

The PVL toxin is a beta-barrel, hetero-oligomeric leukocidin composed of two subunits, the 34 KDa LukF-PV and 33 KDa LukS-PV encoded by the *lukF-PV* and *lukS-PV* genes, respectively. The genes are relatively well conserved with only 12 SNPs identified (O'Hara *et al.*, 2008; Otter *et al.*, 2010) and these 12 SNPs can be used to divide the *lukF/S-PV* genes into two groups (O'Hara *et al.*, 2008). Those with a G instead of A at position 527 in the *lukS-PV* gene are known as the R variant, and those with the original A are known as the H variant of the *lukS/F-PV* operon. The H variants can be subdivided into three groups, H1, H2 and H3 based on further SNPs within the genes.

The two *pvl* genes are co-located on lysogenic prophages belonging to the Siphoviridae family (Kaneko *et al.*, 1998). Thirteen distinct bacteriophages encoding PVL have been described to date including ϕ PVL, ϕ 108PVL, ϕ SLT, ϕ Sa2MW, ϕ Sa2USA, ϕ Sa2958, ϕ SLT-USA300, ϕ TCH1516, ϕ TCH60, ϕ 7247PVL, ϕ 5967PVL and ϕ tp310-1, ϕ IND772 (Kaneko *et al.*, 1998; Narita *et al.*, 2001; Ma *et al.*, 2006; Ma *et al.*, 2008; Wirtz *et al.*, 2010; Boakes *et al.*, 2011; Haddad & Moineau, 2013; Prabhakara *et al.*, 2013). The phage can be subdivided on the basis of isomeric or elongated capsid morphology and host range. During lysogenisation *pvl*-encoding bacteriophage genomes integrate into the *S. aureus* chromosome at one of two chromosomal insertion or attachment sites (*attB*) termed Ins1 and Ins2, both of which contain conserved attachment motifs (Boakes *et al.*, 2012). During lysogenisation, a phage attachment site termed *attP*

recombines with the chromosomal *attB* site followed by integration of the linear phage genome into the bacterial chromosome and duplication of the attachment site sequence at both ends of the integrated phage genome. Site- and orientation-specific recombination is mediated by phage encoded integrase genes. The duplicated attachment sites in the lysogen comprise of two 79 bp sequences, *attL* and *attR*, which are formed from the insertion of *attP* into *attB*. Both the phage and bacterial chromosomal binding sites comprise three regions P1, P2, P3 and B1, B2, B3, respectively. These recombine during insertion of the phage to give the *attR* site B1, B2, P3 and the *attL* site P1, P2, B3 (Boakes *et al.*, 2011). The 29 bp B2 and P2 regions are very similar and share repeat motifs. The chromosomal insertion sites are lineage-specific with Ins1 found in isolates belonging to CC22, CC30, CC80 and Ins2 in isolates belonging to CC1, CC5, CC8, CC59, CC88, ST93 and sometimes CC22. There are possibly other bacteriophage insertion sites that have not yet been identified as in several studies the insertion sites could not be determined using oligonucleotide primers specific for Ins1 or Ins2. The same *pvl*-encoding bacteriophages can be found in a large number of diverse isolates belonging to different lineages; for example ϕ Sa2USA has been found in CC1, CC5, CC8, and ST93 (Table 4.1). While some lineages have to date been found to harbour a unique *pvl*-encoding bacteriophage e.g. ϕ IND772 among ST772 isolates, others have been found to harbour a wide range of different *pvl*-encoding phage e.g. CC5-MRSA isolates have been found to carry ϕ Sa2USA, ϕ pv1, ϕ 108pvl, ϕ TCH60, ϕ Sa2958, ϕ 7247, and ϕ SLT (Table 4.1).

Bacteriophages encoding *pvl* typically comprise five coding regions: 1) a lysogeny segment, encoding site-specific integrase genes; 2) a DNA replication/transcription regulation segment, encoding DNA binding protein, phage replication proteins and a dUTPase; 3) a structural packaging segment, encoding head and tail protein genes including capsid proteins, tail proteins, phage tape measure proteins and other structural proteins; 4) a lysis segment, encoding holin and amidase genes and 5) toxin element, encoding *lukF-PV* and *lukS-PV* genes. The genomes of most *pvl*-encoding bacteriophages are approximately 40 kb in size (Kaneko *et al.*, 1998; Narita *et al.*, 2001; Ma *et al.*, 2006; Ma *et al.*, 2008; Wirtz *et al.*, 2010; Boakes *et al.*, 2011; Haddad & Moineau, 2013; Prabhakara *et al.*, 2013)..

Both the bacteriophage life cycle and host background have been found to be important factors in the expression levels of the PVL toxin (Wirtz *et al.*, 2009). High

expression levels have been linked to increased pathogenesis and low levels linked to improved host innate immune responses to infection. Expression of PVL is highly linked to the success of *pvl*-positive strains (Graves *et al.*, 2012).

New *pvl*-encoding bacteriophages are being found around the world with the emergence and spread of new strains, most recently a novel *pvl*-phage ϕ IND772PVL was discovered in ST772-MRSA-V isolates in India which encoded both *pvl* and the superantigen *sea* encoding enterotoxin A (Prabhakara *et al.*, 2012). This phage was most closely related to ST59-related phages ϕ 7247PVL and ϕ 5967, which are both mosaics and include fragments of an unknown beta-haemolysin (*hly*)- converting phage, the remnants of which encode only *scn* in ST772 (Prabhakara *et al.*, 2012). Uniquely ϕ IND772PVL encodes a fully functional *sea* gene which is highly expressed despite being under the control of a promoter typically associated with low expression levels. Almost 15% of the ϕ IND772PVL phage genes have no ortholog in any other PVL phage. All ST772-MRSA-V isolates investigated to date contain the same ϕ IND772PVL phage, suggesting this unique phage appeared early in the emergence of the ST772 clone in India (Prabhakara *et al.* 2012; Monecke *et al.*, 2013).

With the emergence and spread of strains carrying new *pvl*-encoding phage and the potential for increased virulence of these strains the purpose of this part of the present study was to characterise the genetic organisation of *pvl*-encoding bacteriophages present in a selection of *pvl*-positive MRSA isolates representative of the most commonly identified *pvl*-positive clones in Chapter 3 among isolates submitted to the NMRSARL in Ireland between 2002 and 2012. A total of 10 ST8-MRSA-IV, ST30-MRSA-IV and ST772-MRSA-V isolates underwent whole-genome sequencing and the genetic organisation of *pvl*-encoding prophage genomes were analysed and compared with the corresponding sequences of *pvl*-encoding prophages in GenBank.

Table 4.1 Previously identified *pvl*-encoding bacteriophage and the *S. aureus* genotypes that they have been identified in

<i>pvl</i> -encoding bacteriophage	Genotype	References
φSa2USA	CC1, CC5 ¹ , CC8 ¹ , ST93	Boakes <i>et al.</i> , 2011, Sanchinni <i>et al.</i> , 2014;
φSa2MW	CC1, CC80	Boakes <i>et al.</i> , 2011
φpvl	CC5, CC22, CC30 ¹	Boakes <i>et al.</i> , 2011
φ108pvl	CC5, CC22, CC30 ¹ , ST59, ST239	Hu <i>et al.</i> , 2014; Boakes <i>et al.</i> , 2011;
φInd772	ST772	Prabhakara <i>et al.</i> , 2012
φSa2958	CC5, CC59, CC121 ¹ , ST239	Hu <i>et al.</i> , 2014; Ma <i>et al.</i> , 2008
φ7247	CC5, CC45, CC59, ST239	Hu <i>et al.</i> , 2014; Zhang <i>et al.</i> , 2011
φ5967	CC59	Hu <i>et al.</i> , 2014; Zhang <i>et al.</i> , 2011
φSLT	CC5, CC45, CC88, ST239	Hu <i>et al.</i> , 2014
φTCH60	CC2, CC30, CC88 ¹	Sanchinni <i>et al.</i> , 2014
φSLT-USA 300	CC8	Highlander <i>et al.</i> , 2007
φTCH1516	CC8	Highlander <i>et al.</i> , 2007
φtp310-1	CC22	Reichholf <i>et al.</i> , 2007

¹Within these genotypes *pvl*-encoding bacteriophage have been identified in both MRSA and MSSA, for all other genotypes they have only been identified in MRSA.

4.2 Materials and Methods

4.2.1 Isolates

Chapter 3 describes a detailed investigation of the 276 *pvl*-positive *S. aureus* isolates including 231 MRSA and 45 MSSA identified in Ireland by the NMRSARL between 2002 and 2012. A subset of 10 MRSA isolates were selected from the three main clones identified as part of that study to investigate the comparative genetic organisation of *pvl*-encoding lysogenic bacteriophages. The 10 MRSA were chosen to represent as diverse a selection as possible from the three clones ST8-MRSA-IV, ST30-MRSA-IV and ST772-MRSA-V. Isolates were selected based on differences in antimicrobial resistance and virulence gene carriage as determined by DNA microarray profiling and geographic distribution in Ireland. Four *pvl*-positive ST8-MRSA-IV, three *pvl*-positive ST30-MRSA-IV and three *pvl*-positive ST772-MRSA-V isolates were investigated. Details of these 10 *pvl*-positive MRSA isolates including antimicrobial resistance and virulence gene content as identified by DNA microarray profiling and, where available, epidemiological data, are shown in Tables 4.2-4.4.

4.2.2 Whole-genome sequencing

Whole-genome sequencing was undertaken commercially by Geneservice (Source Bioscience) using the Illumina MiSeq. The reads were assembled into contigs using Velvet *de novo* genome assembler (Version 1.0.15) and contigs were analysed using BioNumerics Software version 6.6 (Applied Math, Ghent, Belgium) and Artemis (Wellcome Trust Sanger). Open reading frames (ORFs) were predicted using Artemis and BioNumerics and all ORFS were checked for consistency between the two programmes and were analysed using BLAST software.

4.2.3 Bacteriophage sequence analysis

Comparison of *pvl*-encoding bacteriophage sequences was carried out using the Artemis comparison tool (ACT) and Bionumerics software. Comparative schematic scale drawings of the genetic organisation of *pvl*-encoding bacteriophages and reference phage sequences obtained from GenBank including ϕ IND772 (Prabhakara *et al.*, 2012), ϕ Sa2958 (accession number AP009363), ϕ 108PVL (accession number AB243556), ϕ 5967PVL (accession number AP011955), ϕ PVL (accession number AB009866),

φSa2USA (accession number CP000255) and φSLT (accession number NC_002661), were prepared using Lucid Chart (Lucid Software Inc. 2015). Multiple sequence alignments were performed using ClustalW2 (Larkin *et al.*, 2007) to compare the *lukF/S-PV* and integrase sequences of the ten *pvl*-positive *S. aureus* isolates.

Table 4.2 Details of ST8-MRSA-IV isolates subjected to whole-genome sequencing to characterise the genetic organisation of *pvl*-encoding bacteriophages present

Isolate name	Year of isolation	Hospital	Region	spa type	Antimicrobial resistance genes ¹										Virulence genes			
					<i>blaZ</i>	<i>msr</i> (A)	<i>mph</i> (C)	<i>aphA3 & sat</i>	<i>sdrM</i>	<i>fosB</i>	<i>mupA</i>	<i>qacC</i>	<i>aacA₂ aphD</i>	<i>tel</i> (K)	<i>sej</i>	<i>sek/q</i>	IEC type ₂	ACME
M06/0057	2006	H4	Dublin	t008	+	+	+	+	+	+	-	-	-	-	+	+	B	+
M11/0104	2011	H5	Dublin	t008	+	+	+	+	+	+	-	-	-	-	-	+	B	+
M09/0149	2009	H3	Dublin	t008	-	+	+	+	+	+	+	-	-	-	-	+	B	+
M09/0145	2009	H1	South-East	t008	+	+	+	+	+	+	-	+	+	-	-	+	B	+

¹+/-, isolates were positive/negative for the gene indicated as identified by the StaphyType DNA microarray (Alere).

²Immune evasion cluster (IEC) types were determined as described previously by Van Wamel *et al.*, (2008). IEC type B: *sak*, *chp* & *scn*.

Table 4.3 Details of ST30-MRSA-IV isolates subjected to whole-genome sequencing to characterise the genetic organisation of *pvl*-encoding bacteriophages present

Isolate name	Year of isolation	Hospital	Region	spa type	Antimicrobial resistance genes ¹						Virulence genes			
					<i>sdrM</i>	<i>fusC</i>	<i>dfrS1</i>	<i>tet</i> (K)	<i>fosB</i>	<i>egc</i>	IEC type ²	<i>sea</i>	<i>tst</i>	
M09/0349	2009	H6	Dublin	t019	+	-	-	-	+	+	A	+	-	
MF181	2004	NA	NA	NA	+	+	-	-	+	+	-	-	+	
M08/0153	2008	H8	Dublin	t318	+	-	+	+	+	+	A	+	-	

¹+/-, isolates were positive/negative for the gene indicated as identified by the StaphyType DNA microarray (Alere). N/A not applicable, data was not collected.

²Immune evasion cluster (IEC) types were determined as described previously by Van Wamel *et al.*, (2008). IEC type A: *sea*, *sak*, *chp* & *scn*.

Table 4.4 Details of ST772-MRSA-V isolates subjected to whole-genome sequencing to characterise the genetic organisation of *pvl*-encoding bacteriophages present

Isolate name	Year of isolation	Hospital	Region	spa type	Antimicrobial resistance genes					Virulence genes						
					blaZ	mst(A)	mph(C)	aacA_aphD	aphA3 &sat	sdrM	sdrD	fosB	sea	sec/I	egc	IEC type ²
M10/0033	2010	H1	South-East	t345	+	+	+	+	+	+	+	+	+	+	+	Novel
M11/0120	2011	H13	Dublin	t657	+	+	+	+	+	+	+	+	+	+	+	Novel
M11/0295	2011	H14	Dublin	t657	+	+	+	+	+	+	+	-	+	+	+	Novel

¹ +/- isolates were positive/negative for the gene indicated as identified by the StaphyType DNA microarray (Alere).

²Immune evasion cluster (IEC) types were determined as described previously by Van Wamel *et al.*, (2008). Novel IEC type: *scn*.

4.3 Results

4.3.1 Characterisation of the *pvl*-carrying phage identified among ten *pvl*-positive MRSA isolates

4.3.1.1 ST8-MRSA-IV isolates

Three different lysogenic phage were identified among the four ST8-MRSA-IV isolates (M06/0057, M11/0104, M09/0149 and M09/0145) investigated (Fig. 4.1 (A)) all of which were integrated into the *ins1* *S. aureus* chromosomal attachment site. The *attR* and *attL* sites among the ST8-MRSA-IV isolates were identical to each other (Tables 4.5 and 4.6). Isolates M09/0149 and M11/0104 harboured identical phage which were related to ϕ Sa2USA which has previously been found in ST8-MRSA-IV isolates (Fig. 4.1 (A) & (D)). The lysogeny modules encoded identical integrase genes to each other, a lipoprotein, repressor protein and regulatory protein. The replication module encoded a DNA polymerase, DNA binding protein, virulence protein E and helicase. The packaging module harboured, similar to the other *pvl* phage in this study, large and small terminase subunits, portal, tail and tape measure proteins and a tail tape measure. The lysis and *lukF/S-PV* regions in both M09/0149 and M11/0104 were identical (Fig. 4.1 (A)). The phage in the remaining two isolates (M06/0057 and M09/0149) were very similar and also related to the ϕ Sa2USA (Fig. 4.1 (A) and (D)). They encoded different sized DNA polymerase genes and the terminase large and small subunits in M06/0057 were in different locations compared to other *pvl*-encoding phage and differed from any previously described *pvl* phage (Fig. 4.1 (A), (D)). The integrase and *pvl* genes of M06/0057 and M09/0149 were indistinguishable (Figs. 4.2-4.4).

4.3.1.2 ST30-MRSA-IV isolates

The three ST30-MRSA-IV isolates (M08/0153, M09/0349 and MF181) harboured two different *pvl*-encoding lysogenic phage (Fig. 4.1 (B)), although both phage were integrated into the same insertion site, *ins2*, in the ST30-MRSA-IV chromosome (Tables 4.5 and 4.6). The three isolates harboured identical *attR* sites (Table 4.6) and similar *attL* sites except for a single base difference at position 6 in the P2 region for isolate M08/0153 with a T instead of a C (Table 4.5). Isolate M08/0153 appeared to have a defective phage related to previously described ϕ PVL (Fig. 4.1 (B) & (D)) which lacked functioning tail proteins (Fig. 4.1 (B)). M08/0153 harboured a shorter *pvl* phage (39.3 kb)

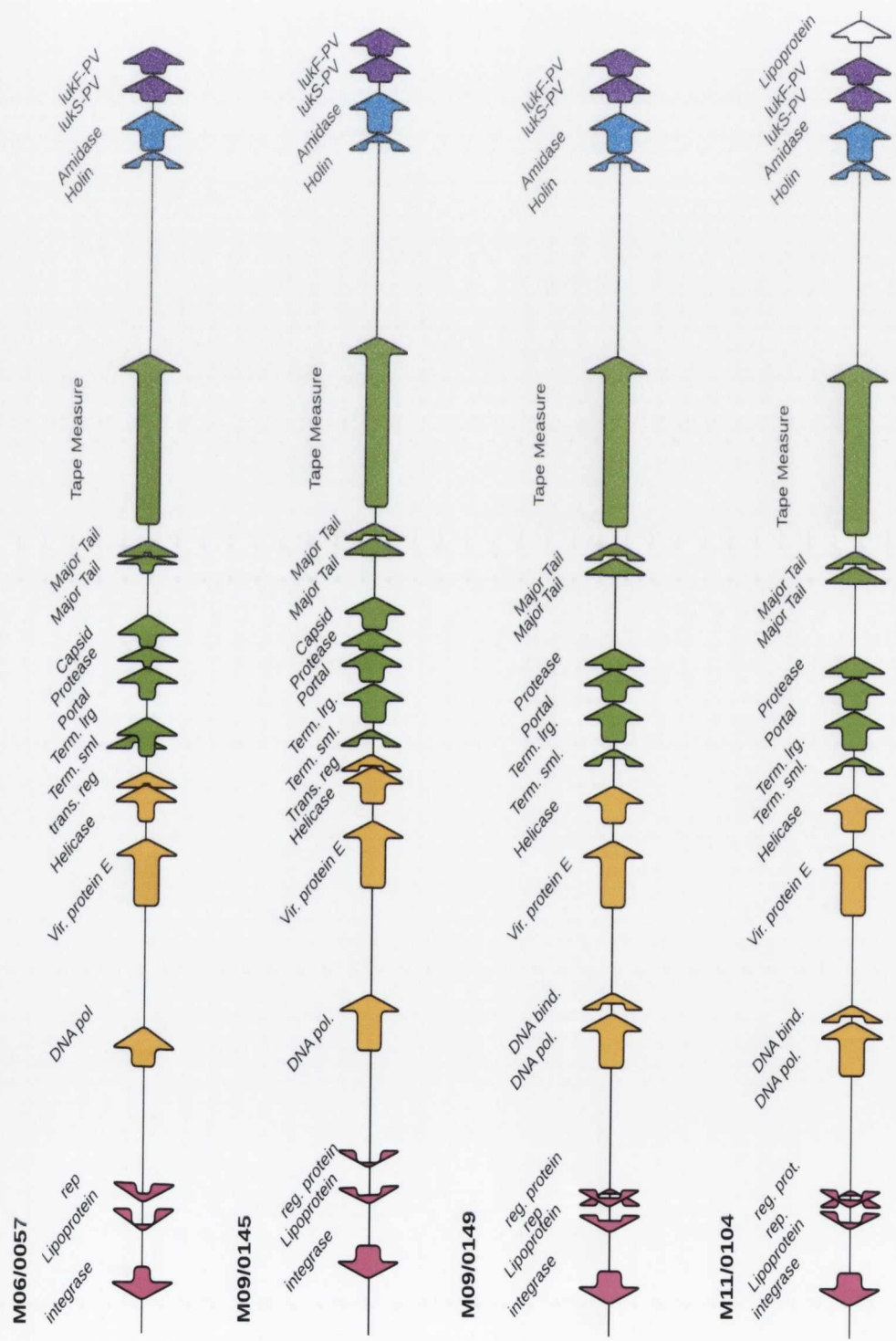
compared to the average 43 kb of other *pvl* phage (Fig. 4.1 (B), (D)). It harboured a lysis module encoding an integrase, repressor protein and anti-repressor. The integrase genes among the three ST30 isolates each differed slightly, M08/0153 was very similar to MF181 with only two nucleotide base differences at positions 1144 (G instead of A, also found among the ST772 *int* gene) and 1156 (A instead of G, unique to M08/0153) (Fig. 4.4). The replication module encoded a ssDNA binding protein and a dUTPase. M08/0153 harboured a packaging module very similar to that found in the other ST30 isolates in the present study with both terminase small and large subunits, and genes for the portal and capsid and a protease, but lacked the entire tail encoding region including the tail tape measure protein. The lysis module consisted of the holin and amidase genes which appeared in all the *pvl*-encoding phage identified and the *lukF/S-PV* genes.

Isolate M09/0349 and MF181 harboured a phage similar to reference phage ϕ Sa2958 (Fig. 4.1 (B) & (D)) which has previously been identified among ST30 isolates (Table 4.1). They differed only in their lysogeny modules. The packaging, lysis modules and *lukF/S-PV* regions were identical to ϕ Sa2958. The integrase gene in M09/0349 harboured seven SNVs compared to MF181, five of these were shared with the ST772 integrase gene (at positions 856, 885, 902, 1030 and 1077, respectively), the other two were unique to M09/0349 and included a T instead of a G at position 229 and a deletion at position 1049 making the M09/0349 integrase gene 1205 bp long compared to the 1206 bp of all the other integrase genes (Fig. 4.4). MF181 harboured an integrase gene identical to that found in the ST8-MRSA-IV isolates (Fig. 4.4).

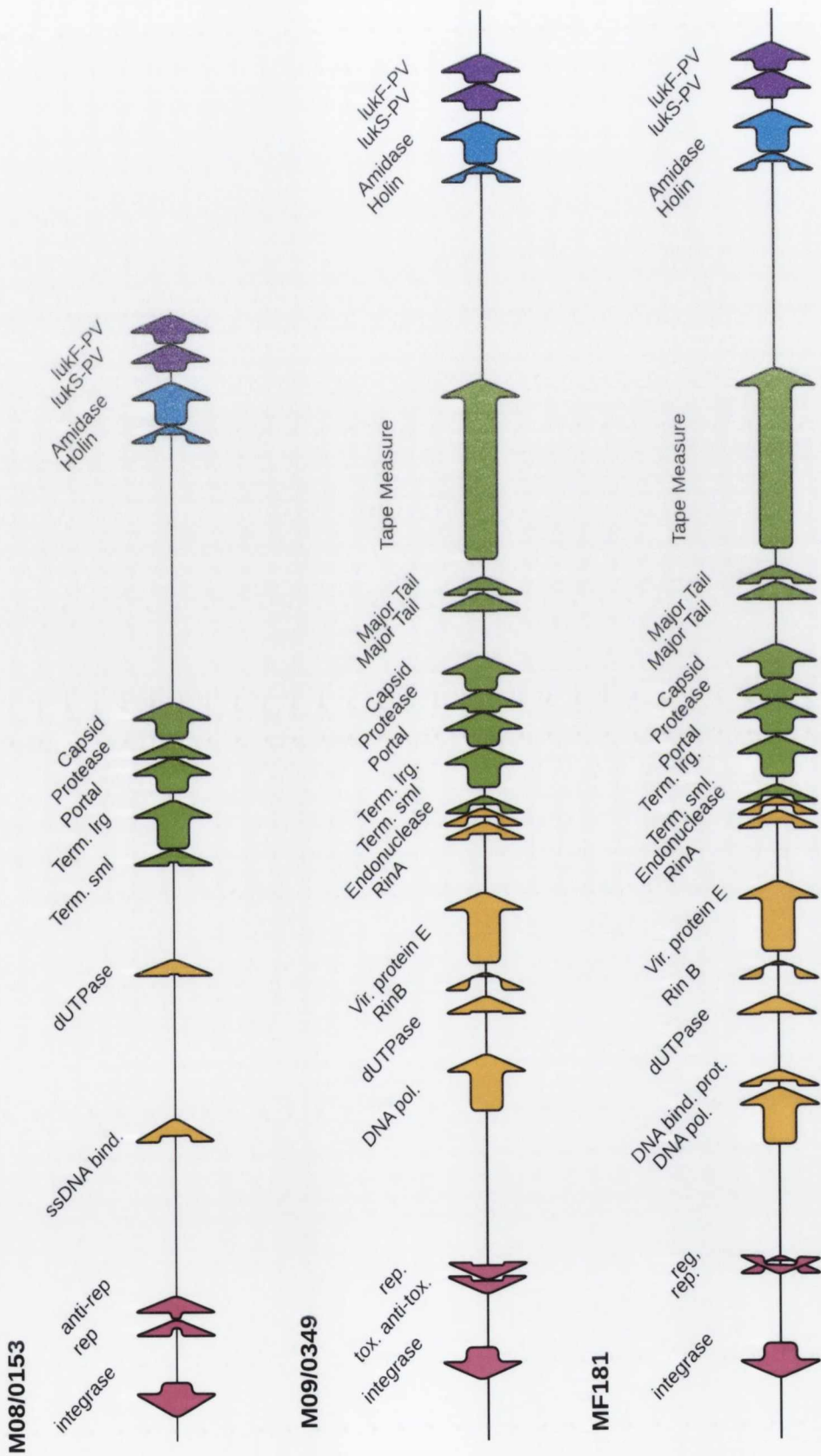
4.3.1.3 ST772-MRSA-V isolates

The three ST772-MRSA-V isolates (M11/0295, M10/0033, M11/0120, Table 4.4) harboured very similar *pvl*-encoding lysogenic phage (Fig. 4.1 (C)). They differed only in sequence of non-coding regions (data not shown). These were similar to previously identified *pvl*-encoding phage in ST772-MRSA isolates from other studies (Fig. 4.1 (C)). The B1, B2 and P3 sites of the attR and the P1, P2 and B3 sites of the attL were identical for each of the *pvl*-encoding phage in the ST772-MRSA-V isolates (Tables 4.5 and 4.6). The three isolates encoded lysogeny modules including integrase genes, a repressor protein, a cro-like repressor and an anti-repressor (Fig. 4.1 (C)). The DNA sequence of the integrase genes among the three ST772-MRSA-V isolates were identical and differed from the integrase genes found among the other seven *pvl*-positive isolates by a total of

(A) *pvl*-encoding phage identified among ST8-MRSA-IV isolates

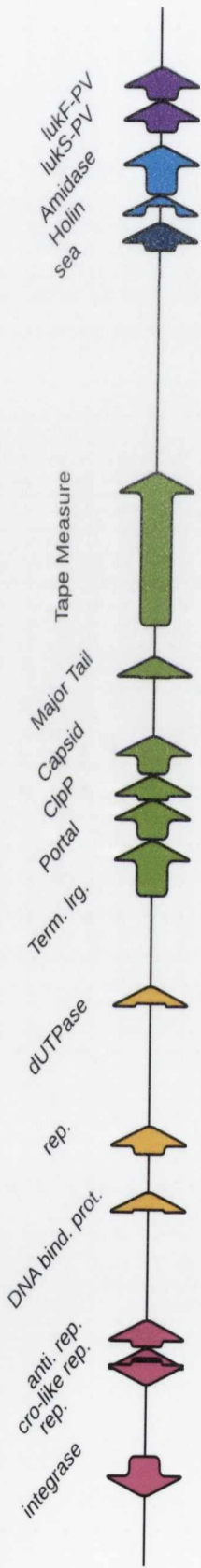


(B) *pvl*-encoding phage identified among ST30-MRSA-IV isolates

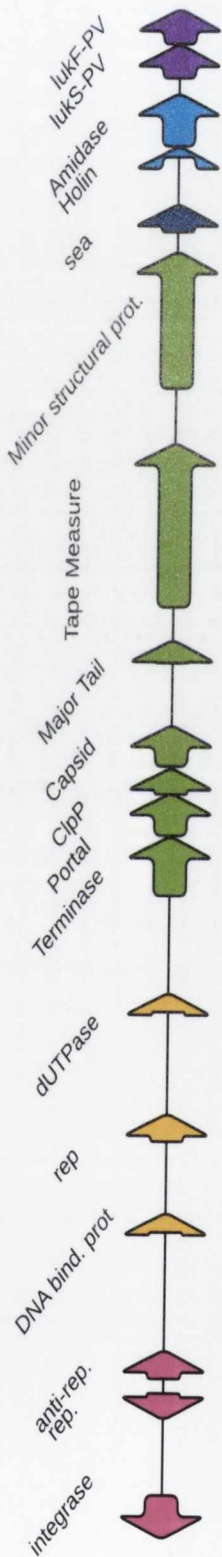


(C) *pvl*-encoding phage identified among ST772-MRSA-V isolates and comparable reference phage

M10/0033, M11/0120,
M11/0295

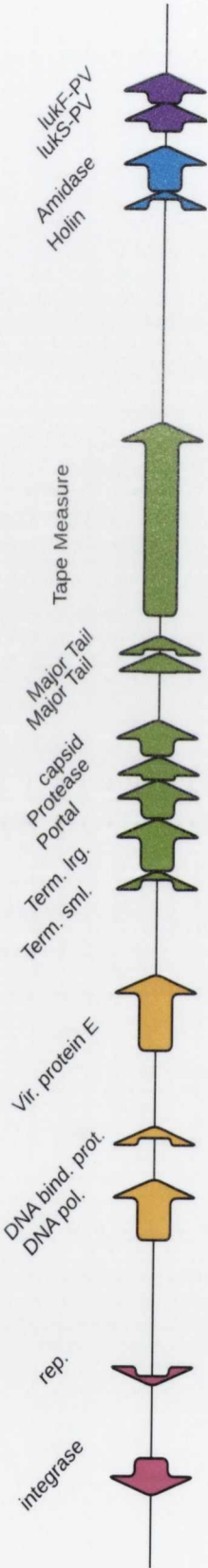


IND772 phage

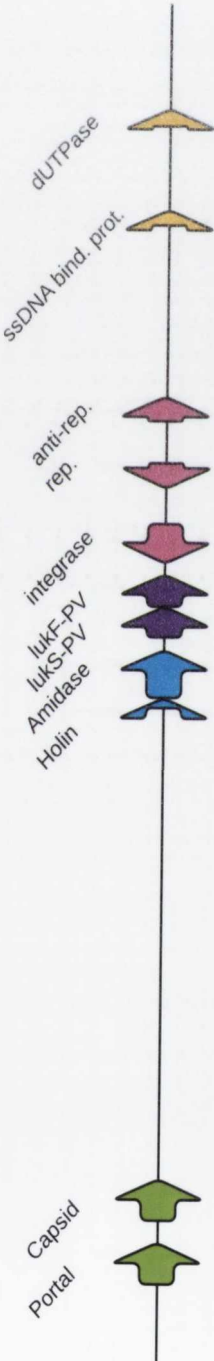


(D) Reference phage associated with phage identified in ST8 (phiSa2USA) and ST30 isolates (phiPVL and phiSa2958) above

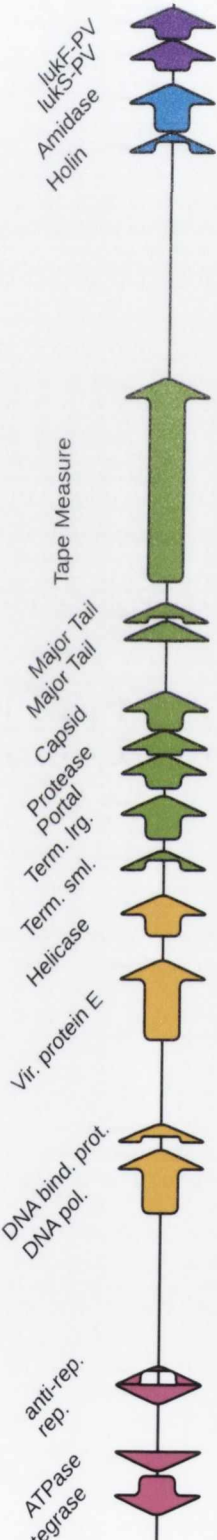
phiSa2USA



phiPVL



phiSa2958



(E) Reference phage

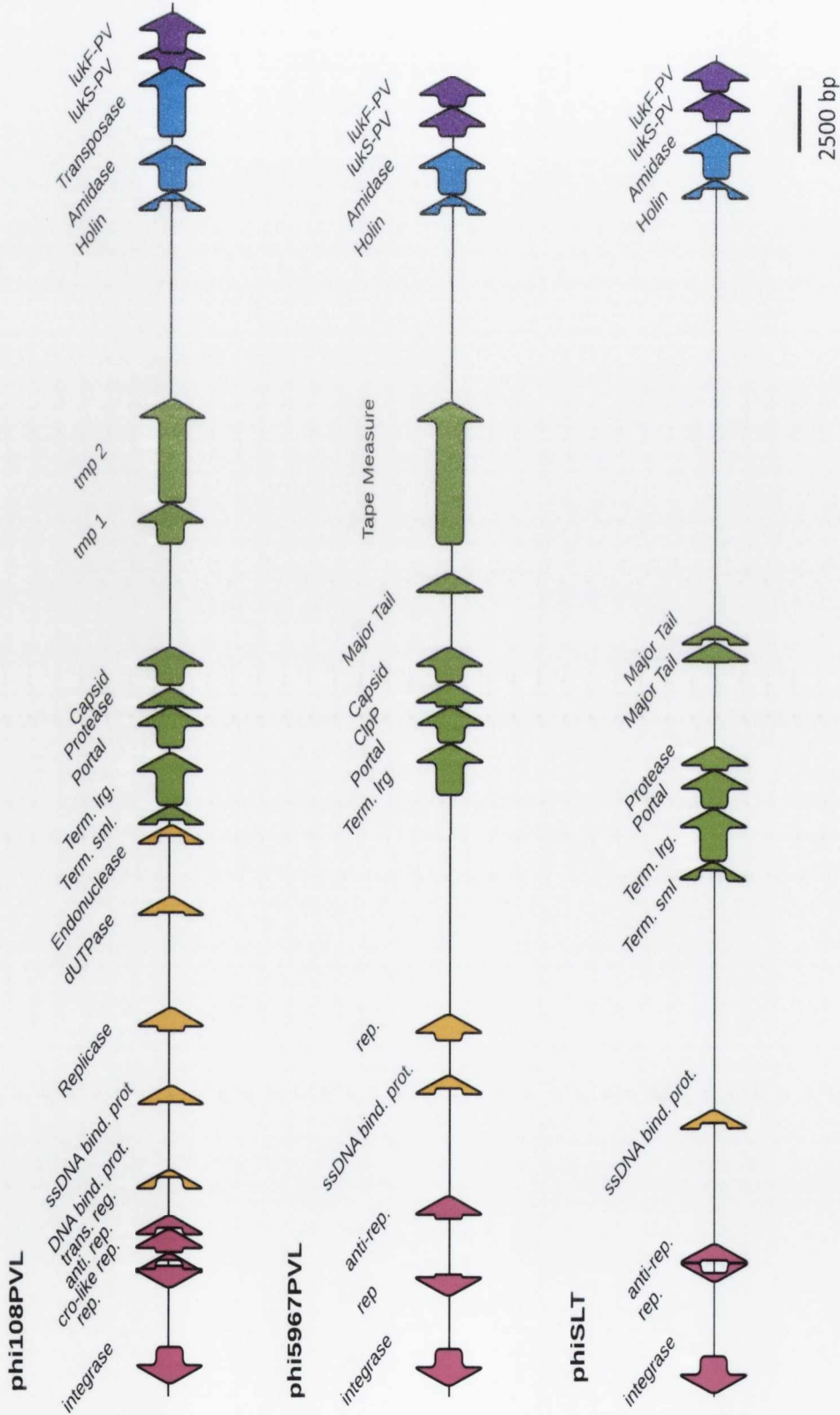


Figure 4.1 Comparison of *pvl*-encoding phage identified by whole-genome sequencing of A) four ST8-MRSA-IV isolates (M06/0057, M09/0145, M09/0149 and M11/0104), B) three ST30-MRSA-IV isolates (M08/0153, M09/0349 and MF181) and C) three ST772-MRSA-V isolates (M11/0295, M10/0033 and M11/0120) recovered from patients in Ireland between 2004 and 2011. Comparable reference phage are also shown, including phage IND772 (Prabhakara *et al.*, 2012), phiSa2958 (AP009363), phi108PVL (AB243556), phi5967PVL (AP011955), phiPVL (AB009866), phiSa2USA (CP000255) and phiSLT (NC_002661)). Genes highlighted in pink represent the lysogeny module of the phage, genes highlighted in yellow represent the replication module, genes highlighted in green are involved in packaging, and encode head and tail proteins, genes highlighted in light blue represent the lysis module, those in dark blue are the enterotoxin A gene, genes highlighted in purple represent the *pvl*-encoding genes *lukS-PV* and *lukF-PV* and genes in white are additional lipoproteins which appear after the *lukF/S-PV* genes in some phage. Abbreviations: *rep.*, repressor; DNA bind. prot., DNA binding protein; term. lrg./sml, terminase large/small; Vir. Protein E, virulence protein E.



Figure 4.2 Regions of DNA sequence variation identified following a multiple sequence alignment of *lukF-PV* genes from the 10 *pvl*-positive MRSA isolates investigated that were recovered from patients in Ireland between 2004 and 2011 belonging to genotypes ST8-MRSA-IV (M06/0057, M11/0104, M09/0149 and M09/0145), ST30-MRSA-IV (M09/0349, MF181 and M08/0153) and ST772-MRSA-V (M10/0033, M11/0120 and M11/0295). Coloured bases highlight sequence variation within the specified isolate. The *lukF-PV* gene is 978 base pairs in length and 120 base pairs of this are shown.

M06/0057	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M11/0104	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M09/0149	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M09/0145	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M09/0349	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
MF181	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M08/0153	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATATAATTATTCA	480
M10/0033	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M11/0120	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480
M11/0295	ATAGGTGGTAATTTTAATAGTGGTCCATCAACAGGAGGTAATGGTTCATTTAATTATTCA	480

M06/0057	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACGTCAAAATTCAAAA	540
M11/0104	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACGTCAAAATTCAAAA	540
M09/0149	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACGTCAAAATTCAAAA	540
M09/0145	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACGTCAAAATTCAAAA	540
M09/0349	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540
MF181	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540
M08/0153	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540
M10/0033	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540
M11/0120	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540
M11/0295	AAAACAATTAGTTATAATCAACAAAACATATATCAGTGAAGTAGAACATCAAAAATTCAAAA	540

M06/0057	GTTCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M11/0104	GTTCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M09/0149	GTTCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M09/0145	GTTCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M09/0349	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
MF181	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M08/0153	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M10/0033	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M11/0120	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
M11/0295	GTGCCAGACAATGAATTACCCCCATTAGTACACAGTGGTTTCAATCCTTCATTTATTGCA	720
** *****		

Figure 4.3 Regions of variation identified in a multiple sequence alignment of *lukS-PV* genes from the ten *pvl*-positive MRSA isolates investigated that were recovered from patients in Ireland between 2004 and 2011 belonging to genotypes ST8-MRSA-IV (M06/0057, M11/0104, M09/0149 and M09/0145), ST30-MRSA-IV (M09/0349, MF181 and M08/0153) and ST772-MRSA-V (M10/0033, M11/0120 and M11/0295). Coloured bases highlight sequence variation within the specified isolate. The *lukS-PV* gene is 939 base pairs in length and 180 base pairs of this are shown.

M06/0057 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M11/0104 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M09/0149 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M09/0145 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M09/0349 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
MF181 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M08/0153 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTTTTTTCAGTTACATG 60
M10/0033 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTCTTTCAGTTACATG 60
M10/0120 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTCTTTCAGTTACATG 60
M11/0295 CTAACCTGGAATGTTTTTCGAGTTTTTGAGTTAGTTCTCTATCCATTCTTTCAGTTACATG 60

M06/0057 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M11/0104 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M09/0149 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M09/0145 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M09/0349 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
MF181 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M08/0153 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M10/0033 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M10/0120 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120
M11/0295 AGTATATATGCGAATGGTTGTTTTTTCATCTACATGTCCTACCCTTTTCATAATTGCTTT 120

M06/0057 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M11/0104 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M09/0149 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M09/0145 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M09/0349 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
MF181 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M08/0153 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M10/0033 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M10/0120 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240
M11/0295 AACTTTCCTTATTCATATTTAAAGCTTTTGTAGTTTTCTTAAGCACACCGGCGATTTGATT 240

M06/0057 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M11/0104 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M09/0149 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M09/0145 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M09/0349 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
MF181 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M08/0153 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M10/0033 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M10/0120 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300
M11/0295 ATTACATAAAGGATTCCTTTTTTTTGTGTGAATATGAACCCCTCTGTCAACATAGCTCGA 300

M06/0057 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M11/0104 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M09/0149 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M09/0145 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M09/0349 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
MF181 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M08/0153 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M10/0033 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M10/0120 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420
M11/0295 AGAATTGATGCTGATACTTCTTTTTGAACTTATAGTCTTTGTAGTGTCTTTGTATCCGAA 420

M06/0057 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M11/0104 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M09/0149 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M09/0145 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M09/0349 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
MF181 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M08/0153 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M10/0033 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480
M10/0120 TCCTTCCTCGTATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTAATAA 480

M11/0295 TCCTTC**TTC**ATATTTAATGCGGTGAATTGTACCTGTTATATTGATAGTTTTGTTTAATAA 480

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 M09/0149 ATCTATATCTTTTTCTGCAGTGCTTGTAGTTCTCCTATGCGCATACCAGTTAAAGCCTG 540
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 M09/0349 ATCTATATCTTTTTCTGCAGTGCTTGTAGTTCTCCTATGCGCATACCAGTTAAAGCCTG 540
 MF181 ATCTATATCTTTTTCTGCAGTGCTTGTAGTTCTCCTATGCGCATACCAGTTAAAGCCTG 540
 M08/0153 ATCTATATCTTTTTCTGCAGTGCTTGTAGTTCTCCTATGCGCATACCAGTTAAAGCCTG 540
 M10/0033 ATCTATATCTTTTTCTG**AA**GTGCTTGTA**ATT**CTCCTATGCGCATACCAGTTAAAGCCTG 540
 M10/0120 ATCTATATCTTTTTCTG**AA**GTGCTTGTA**ATT**CTCCTATGCGCATACCAGTTAAAGCCTG 540
 M11/0295 ATCTATATCTTTTTCTG**AA**GTGCTTGTA**ATT**CTCCTATGCGCATACCAGTTAAAGCCTG 540

M06/0057 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
 M11/0104 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
 M09/0149 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
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 M09/0349 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
 MF181 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
 M08/0153 TACTTCTAAGATGCTGGCAATTAATAATGCGATTCGCTTGTGTAACCTATTATCATTTAG 600
 M10/0033 TACTTCTAAGAT**ACT**GGCAATTAATAATGCGATTCGCTTGTGTAACCTATTAT**T**ATTTAG 600
 M10/0120 TACTTCTAAGAT**ACT**GGCAATTAATAATGCGATTCGCTTGTGTAACCTATTAT**T**ATTTAG 600
 M11/0295 TACTTCTAAGAT**ACT**GGCAATTAATAATGCGATTCGCTTGTGTAACCTATTAT**T**ATTTAG 600

M06/0057 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M11/0104 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M09/0149 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M09/0145 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M09/0349 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 MF181 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M08/0153 ATCTTTTTCGATATCCTCTATTGTTTTCTTCTTTTAGGAATTTTGACATTAGTTAACAA 720
 M10/0033 ATCTTTTTCGATATC**TT**CTATTGTTTTCTTCTTTTAGG**T**ATTTTGACATTAGTTAACAA 720
 M10/0120 ATCTTTTTCGATATC**TT**CTATTGTTTTCTTCTTTTAGG**T**ATTTTGACATTAGTTAACAA 720
 M11/0295 ATCTTTTTCGATATC**TT**CTATTGTTTTCTTCTTTTAGG**T**ATTTTGACATTAGTTAACAA 720

M06/0057 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M11/0104 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M09/0149 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
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 M09/0349 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 MF181 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M08/0153 ATATTCAATTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M10/0033 ATATT**C**TTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M10/0120 ATATT**C**TTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780
 M11/0295 ATATT**C**TTTGGATAAATTGTAAATTTAACTGCATATTTAATAGCTCCTTTTCATATCTCC 780

M06/0057 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M11/0104 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M09/0149 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M09/0145 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M09/0349 CATATATTTTGTATC**AA**GTTTGTTTAAAAGCAAGTTCTCAGAGC**AG**TATCGTTTAATGTT 900
 MF181 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M08/0153 CATATATTTTGTATCTAGTTTGTTTAAAAGCAAGTTCTCAGAGCTGTATCGTTTAATGTT 900
 M10/0033 CATATATTTTGTATC**AA**GTTTGTTTAAAAGCAAGTTCTCAGAGC**AG**TATCGTTTAATGTT 900
 M10/0120 CATATATTTTGTATC**AA**GTTTGTTTAAAAGCAAGTTCTCAGAGC**AG**TATCGTTTAATGTT 900
 M11/0295 CATATATTTTGTATC**AA**GTTTGTTTAAAAGCAAGTTCTCAGAGC**AG**TATCGTTTAATGTT 900

M06/0057 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M11/0104 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M09/0149 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M09/0145 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M09/0349 **T**TAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 MF181 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M08/0153 TCTAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960
 M10/0033 **T**TAATTCCTATTTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTATATG 960

M10/0120	TTTAATTCTTATTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTTATATG	960
M11/0295	TTTAATTCTTATTTTATATTATTAAGAGTAGTCAACTTTGAACCTGATGTTTTTATATG	960
	* * * * *	
M06/0057	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M11/0104	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M09/0149	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M09/0145	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M09/0349	CTTGTTGTTT AG TTTTTCTTTTATTTTT-CTTCTAAACGAAACATTGCTTCTTTT G TGA	1079
MF181	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M08/0153	CTTGTTGTTTCAGTTTTTCTTTTATTTTTCTTCTAAACGAAACATTGCTTCTTTTTGAGA	1080
M10/0033	CTTGTTGTTT AG TTTTTCTTTTATTTTTCTTCTAAAC G AACAT AG CTTCTTTT G TGA	1080
M10/0120	CTTGTTGTTT AG TTTTTCTTTTATTTTTCTTCTAAAC G AACAT AG CTTCTTTT G TGA	1080
M11/0295	CTTGTTGTTT AG TTTTTCTTTTATTTTTCTTCTAAAC G AACAT AG CTTCTTTT G TGA	1080
	* * * * *	
M06/0057	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M11/0104	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M09/0149	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M09/0145	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M09/0349	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1139
MF181	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M08/0153	TTGTTTTGTATTCTTGTTCAACACAACACTTACGCGCTTCCATTTATCTGTGTATGGATC	1140
M10/0033	TTGTTTTGTATT TT TGTTCAAGACA TA CTTAC ACG TTTCCATTTATCTGTGTATGGATC	1140
M10/0120	TTGTTTTGTATT TT TGTTCAAGACA TA CTTAC ACG TTTCCATTTATCTGTGTATGGATC	1140
M11/0295	TTGTTTTGTATT TT TGTTCAAGACA TA CTTAC ACG TTTCCATTTATCTGTGTATGGATC	1140
	* * * * *	
M06/0057	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M11/0104	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M09/0149	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M09/0145	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M09/0349	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1199
MF181	TTTATACTTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M08/0153	TTT G TA CT TTCTCGTA A TATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M10/0033	TTT G TA CT TTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M10/0120	TTT G TA CT TTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
M11/0295	TTT G TA CT TTCTCGTAGTATCTGTATTTAGTTTCGTTATTTTTGTTTTTAAATTTTTCAAT	1200
	* * * * *	
M06/0057	CCACAT	1206
M11/0104	CCACAT	1206
M09/0149	CCACAT	1206
M09/0145	CCACAT	1206
M09/0349	CCACAT	1205
MF181	CCACAT	1206
M08/0153	CCACAT	1206
M10/0033	CCACAT	1206
M10/0120	CCACAT	1206
M11/0295	CCACAT	1206
	* * * * *	

Figure 4.4 Regions of variation from a multiple ClustalW comparison alignment of the *pvl*-encoding phage integrase genes from four ST8-MRSA-IV isolates (M06/0057, M11/0104, M09/0149 and M09/0145,), three ST30-MRSA-IV isolates (M09/0349, MF181 and M08/0153) and three ST772-MRSA-V isolates (M10/003, M11/0120 and M11/0295) recovered from patients in Ireland between 2004 and 2011. Asterisks (*) denote regions of a fully conserved base among all isolates. Bases highlighted in red represent variation. – represents deletion of base.

Table 4.5 Sequence of the *attL* site for *pvl*-encoding phage identified within 10 *pvl*-positive MRSA isolates recovered from patients in Ireland between 2004 and 2011

Sequence type- SCC <i>mec</i> type	Isolate number	<i>attL</i> sequence ¹
ST8-MRSA-IV	M06/0057	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
	M11/0104	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
	M09/0149	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
	M09/0145	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
ST30-MRSA-IV	M08/0153	GCGTACAACGACGAAATGTCAAATTAACCATTCATTATGATGATATGTTTATTTTAAACACTCAAGCTCATGCACGTCT
	M09/0349	GCGTACAACGACGAAATGTCAAATTAACCATCTCATTATGATGATATGTTTATTTTAAACACTCAAGCTCATGCACGTCT
	MF181	GCGTACAACGACGAAATGTCAAATTAACCATCTCATTATGATGATATGTTTATTTTAAACACTCAAGCTCATGCACGTCT
ST772-MRSA-V	M10/0033	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
	M11/0120	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT
	M11/0295	GCGTACAACGACGAAATGTCAAATTAACCATCACATTATGACGATATGTTTATTTTAAACACACAAGCTCATGCGCGTCT

¹ Bases highlighted in blue represent the P1 region, those in black represent the P2 region, those in green the B3 region of the *attL* site and the base in red is the only instance of sequence diversity within a sequence type.

Table 4.6 Sequence of the *attR* site for *pvl*-encoding phage identified within 10 *pvl*-positive MRSA isolates recovered from patients in Ireland between 2004 and 2011

Sequence type-SCCmec type	Isolate number	<i>attR</i> sequence ¹
ST8-MRSA-IV	M06/0057	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
	M11/0104	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
	M09/0149	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
	M09/0145	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
ST30-MRSA-IV	M08/0153	CATTAATTGTTAATCAAAATTTTGAACCATCTCATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
	M09/0349	CATTAATTGTTAATCAAAATTTTGAACCATCTCATTATGATGATATTTTAATTTCAGAAAAAGCTTTAACGCCAGTGTT
	MF181	CATTAATTGTTAATCAAAATTTTGAACCATCTCATTATGATGATATGTTATTTCAGAAAAAGCTTTAACGCCAGTGTT
ST772-MRSA-V	M10/0033	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATAATTAGTCCAGAAAAATCTTTAATGCCAGTGTT
	M11/0120	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATAATTAGTCCAGAAAAATCTTTAATGCCAGTGTT
	M11/0295	CTTTGATTGTACAAATGATATTTGAACCATCACATTATGATGATATAATTAGTCCAGAAAAATCTTTAATGCCAGTGTT

¹Bases highlighted in blue represent the B1 region, those in black represent the B2 region, those in green the P3 region of the *attR* site and the bases highlighted in red are the sequence diversity within sequence types.

30 bases (Fig. 4.4), they were identical to the integrase genes found in the previously described ϕ IND772 phage (sequence data not shown). Twenty-four of these 30 bases were unique to the ST772 integrase gene and six were shared with one other isolate (five in M09/0349 (ST30-MRSA-IV) and one in M08/0153 (ST30-MRSA-IV)) (Fig. 4.4). The three ST772 isolates' *pvl* phage replication module encoded a DNA binding protein, a repressor protein and a dUTPase which were identical (sequence data not shown) to those previously described in phage ϕ IND772 (Fig. 4.4). The packaging module encoded portal, capsid and major tail proteins in addition to identical tape measure proteins and the protease ClpP, as well as a large terminase subunit similar to the ϕ IND772 phage previously identified (Fig. 4.1 (C)). However, in contrast to other *pvl*-encoding phage there was no small terminase subunit. Uniquely, the *pvl* phage detected in the ST772 isolates in the present and previous studies harboured the enterotoxin A gene *sea*, which was located after the packaging module just prior to the lysis module encoding holin and amidase genes and finally the *lukS-PV* and *lukF-PV* genes (Fig. 4.1-4.3).

4.3.2 Analysis of *lukF-PV* and *lukS-PV* genes encoded by the ten *pvl*-positive MRSA isolates.

To compare the *lukF-PV* and *lukS-PV* genes present among the ten isolates investigated a ClustalW analysis of the genes was performed (Figs. 4.2 and 4.3). The four ST8-MRSA-IV isolates (M06/0057, M11/0104, M09/0149 and M09/0145) harboured identical *lukF-PV* (978 bp) and *lukS-PV* (939 bp) genes, but they differed from the corresponding genes in the other isolates at three positions (Fig. 4.2 and 4.3). In *lukF-PV* the ST8-MRSA-IV isolates had a G instead of an A at position 789, in *lukS-PV* they all carried a G instead of an A at position 527 and a T instead of a G (663).

All three ST30-MRSA-IV isolates harboured different *lukF-PV* genes, each differing by a single base (Fig. 4.2). Both M09/0349 and MF181 carried identical *lukS-PV* genes and M08/0153 differed by a single base (A instead of T) at position 470 (Fig. 4.3).

The three ST772-MRSA-V isolates encoded identical *lukF-PV* and *lukS-PV* genes (Figs. 4.2 and 4.3).

The SNVs observed in *lukF-PV* and *lukS-PV* in the 10 isolates investigated divided the isolates into two groups, H (M11/0295, M11/0120 & M10/0033 (ST772); M09/0349 & M08/0153 & MF181 (ST30)) and R (M06/0057, M11/0104, M09/0149 &

M09/0145 (ST8)) variants. The six isolates harbouring the H variant were found to belong to H1 and H2. MF181 (ST30) was the only H1 variant identified with a G instead of an A at position 476. The remaining isolates (M11/0295, M11/0120 & M10/0033 (ST772); M09/0349 & M08/0153 (ST30)) all belonged to H2.

4.4 Discussion

In recent years *pvl*-positive MRSA have received significant attention due to the epidemiological link between *pvl*-positive clones and the severe manifestations of MRSA disease in the community. While the whole-genomes of a relatively small number of *pvl* phage have been published, the majority of studies have utilised PCRs targetting specific phage regions to characterise the genetic organisation of *pvl* phage from MRSA isolates from diverse genetic backgrounds (Boakes *et al.*, 2011, Chen *et al.*, 2013). While these PCR-based studies have provided valuable insights into the genetic organisation of *pvl* phage within different MRSA lineages, it is only by using WGS that the entire genetic organisation of these phage can be definitively deduced. The present study has investigated the relationships between the *pvl*-encoding phage, the phage structure, their integration sites and the *pvl* toxin variants in a selection of isolates from the most commonly identified *pvl*-positive *S. aureus* clones in Ireland between 2002 and 2012 using whole-genome sequencing. The isolates investigated were also representative of major pandemic CA-MRSA clones, namely USA300 (ST8-MRSA-IV), the South West Pacific clone (ST30-MRSA-IV) and the Bengal Bay clone (ST772-MRSA-V) (Monecke *et al.*, 2011). This study has provided further evidence of the diversity of *pvl*-phage among the former two clones, and the presence of a unique *pvl*-phage among isolates of Bengal Bay clone.

A total of six diverse *pvl*-encoding phage were identified among the ten *pvl*-positive MRSA isolates investigated. The three *pvl*-encoding phage identified among the ST8-MRSA-IV isolates ($n = 4$) and the two identified among the ST30-MRSA-IV isolates ($n = 3$) were similar to those described previously among isolates of these genotypes. The structural and lysis modules from three of the ST8-MRSA-IV (M09/0149, M11/0104 and M09/0145) isolates phage and two of the ST30 isolates (M09/0349 and MF181) had very similar genetic arrangements, they differed only in the presence of capsid genes which were missing for two of the ST8-MRSA-IV isolates (M09/0149 and M11/0104). The diversity of *pvl* phage identified among these isolates is not suprising given the results of our previous study into *pvl*-positive *S. aureus* in Ireland (chapter 3) which identified high level of genotypic diversity among clones, and multiple importations of clones into Ireland which could be linked to travel and immigration of individuals from across the globe (Chapter 3).

The three ST772-MRSA-V isolates investigated as part of the present study had very similar DNA microarray profiles, with just one isolate (M11/0295) differing in the detection of *sdrD* and *ccr* genes, which it lacked. Another ST772-MRSA-V isolate (M10/0033) exhibited *spa* type t345 closely related to t657 commonly associated with ST772. Despite these differences the *pvl* phages present among these three isolates were identical in their coding regions, further evidence for the theory that this unique phage emerged early in the existence of the ST772-MRSA-V strain and the diversity detected using the DNA microarray developed after this. Staphylococcal enterotoxin A is a potent superantigen which, unlike other antigens, is capable of stimulating up to 30% of T cells in the host, leading to a massive cytokine storm (Deringer *et al.*, 1996). The enterotoxin A gene *sea* is normally found encoded on *hly*-converting phages which harbour various combinations of the immune evasion complex (IEC) genes *sea*, *sak*, *chp*, *scn*, and *selp*; within these *hly*-converting phages *sea* is located adjacent to the structural module. It has been hypothesised that a single recombination event led to the transfer of the structural capsid and tail genes and *sea* from a *hly*-converting phage to a *pvl* phage in ST772-MRSA-V (Prabhakara *et al.*, 2013). The remnant of the *hly*-converting phage in ST772-MRSA-V strains encodes *scn*, the latter normally encoded by *hly*-converting phages. This recombination event most likely occurred early in the emergence of ST772-MRSA-V strains as this phage has been identified in all ST772-MRSA-V investigated to date, some of which, have been from disparate geographic locations including India, Germany and Ireland (Monecke *et al.*, 2013; Prabhakara *et al.*, 2013). The presence of genes encoding a potent superantigen and a cytolytic toxin (*pvl*) on a single phage raises concerns for the possible spread of this phage into other very successful pandemic MRSA clones such as ST8-MRSA-IV potentially causing more serious and life-threatening infections, although no evidence of this was detected in the present study.

The *pvl*-encoding phage identified inserted into one of two insertion sites, with some inter- and intra-lineage variation present. The *attL* and *attR* sites for the ST8-MRSA-IV isolates investigated here were identical and had both been identified previously in isolates from CCs 1, 8, 93 and 5 (Boakes *et al.*, 2010). The *attL* sites from the *pvl*-encoding phage among the ST772-MRSA-V isolates were identical to those found in the ST8-MRSA-IV isolates in the present study, whereas the *attR* sites were unique. The P3 regions of the *attR* sites identified in the ST772 isolates were identical to those previously found in isolates from CC5, the last 8 bases from B2 were also similar to

regions identified in CC5 isolates (Boakes *et al.*, 2010). Region B1 showed similarities to an *attR* site identified in CC5 isolates though the three regions had not been identified in CC5 isolates together. The *attR* site of the novel ST772 phage had previously been shown to be similar to that of phage 7247 identified in ST59-MRSA isolates which also harbours an *attL* site identical to that found in ST8-MRSA-IV isolates (Prabhakara *et al.*, 2012). Within the ST30-MRSA-IV isolates, two *attR* and *attL* combinations were identified, both of which had previously been associated with CC30 isolates (Boakes *et al.*, 2010). The main variation in the *attR* region is located on the 29bp B2 fragment indicating the diversity of *attR* sequences are due to differences in the chromosomal DNA of the integration site (Chen *et al.*, 2013). These results indicate that the two insertion sites for *pvl*-phage are strongly maintained within clones from disparate geographic locations and no new sites were identified here.

A total of six SNVs were identified among the *lukS-PV* and *lukF-PV* genes of the ten isolates, all of which had been described previously (Otter *et al.*, 2010; Berglund *et al.*, 2008; Wolter *et al.*, 2007). The only SNV encoding an amino acid change was in *lukS-PV* where the G instead of an A in position 527 leads to an arginine instead of a histidine (Wolter *et al.*, 2007). This SNV defines the R variants which all belonged to ST8-MRSA-IV. The R variants are associated with more recent CA-MRSA strains and have been identified in ST8-MRSA-IV strains previously (O'Hara *et al.*, 2008; Wolter *et al.*, 2007). The R variant was reported as far back as 1986 before the emergence of most CA-MRSA strains and it is hypothesised that ST8-MRSA-IV acquired the R variant in phage Sa2USA from a CC30-MSSA strain (Chen *et al.*, 2013). The H variants harbouring the original histidine were subdivided into two further groups, H1 and H2. H variants are associated with European clones such as ST80 and ST30, however both the H1 and H2 variants have been found in South Africa and India (O'Hara *et al.*, 2008). The H1 and H2 variants were identified in isolates from ST30-MRSA in the present study, all the ST772-MRSA-V isolates, associated with India, harboured the H2 variant of the *lukF/S-PV*.

The G instead of an A in position 527 amino acid change in *lukS-PV* increases the predicted molecular size from 35294 Da to 35313 Da and this change has been hypothesised to be linked to the increased disease severity seen among CC/ST8-MRSA-IV isolates (O'Hara *et al.*, 2008). A direct functional difference between the two variants has yet to be proven however and this study previously identified no increased disease severity among CC8-MRSA-IV isolates compared to other *pvl*-positive isolates (Chapter

3). It is possible that other factors such as hospital infection policies, nasal carriage and variations in human populations play a role in the differing disease profiles seen in some studies.

In conclusion, this study has highlighted the diversity of *pvl*-encoding phage among the two major pandemic MRSA clones, ST30-MRSA-IV and ST8-MRSA-IV, as well as a unique clone among the pandemic ST772-MRSA-V clone, all of which predominate among *pvl*-positive MRSA in Ireland. The presence of *pvl* phage with different genetic organisations within a single clone provides evidence for the horizontal transmission of phage within sub lineages of dominant clones and it also provides further evidence of the multiple independent acquisitions of *pvl*-encoding phage by a clone possibly by the importation of new strains into the country. The presence of a novel *pvl*-encoding phage among the ST772-MRSA-V isolates which evolved from a recombination event between a *hly*-converting phage and led to the emergence of the *pvl*-phage which contains *sea* is a worrying development. The potential exists for another recombination event to transfer the *pvl* gene to a full *hly*-converting phage which could potentially lead to the IEC cluster, *sea* and *pvl* existing on one highly virulent and transferable mobile genetic element. The limitations of this study include the fact that only a limited selection of isolates from the three *pvl*-positive clones in Ireland were investigated. In recent years the numbers of *pvl*-positive ST22-MRSA-IV isolates have been increasing in Ireland (Chapter 3), this study did not investigate any isolates of this clone. The *pvl*-positive MSSA which are more diverse than the MRSA in the country (Chapter 3) were also not investigated and the potential exists for further unknown phage to be identified in these populations.

Chapter 5

MRSA and MSSA from Bloodstream infections in Ireland

5.1 Introduction

Staphylococcus aureus is the second most common cause of BSIs in Ireland accounting for approximately 21% of those monitored from patients in Irish hospitals in 2013 (Table 5.1). The ability of *S. aureus* to cause such serious infections is due in part to the extensive repertoire of virulence factors that it can produce including toxins, evasins and adhesions that aid in attachment and damage to host tissue and cells and evasion of the immune system (Otto, 2013). In addition, the ability of *S. aureus* to develop resistance to many different antimicrobial agents, often due to the acquisition of antimicrobial resistance determinants on MGEs, complicates treatment options for *S. aureus* BSIs with MRSA being linked to more persistent BSIs than MSSA and with a higher mortality rate (Chong *et al.*, 2013; Kaasch *et al.*, 2014). However, an increase in mortality rates for MSSA BSIs have been reported in recent years (Harbarth *et al.*, 1998; Tom *et al.*, 2014). An overall mortality rate up to 30% has been reported among patients with MRSA and MSSA BSIs (Kaasch *et al.*, 2014). Bloodstream infections due to MRSA are more often associated with older patients and those with underlying health problems and HCA risk factors than patients with MSSA infections (Chen *et al.*, 2013; Levesque *et al.*, 2014). Reported predictive factors for mortality among patients with MRSA BSIs include age >70 years, a high-risk source such as the presence of a catheter or HCA-wound infection, and initial treatment with inappropriate antimicrobial agents (Gasch *et al.*, 2012).

The epidemiology of *S. aureus* invasive infections has undergone dramatic change in the last twenty years. Several studies have noted a shift in the roles of MRSA and MSSA in invasive infection with MRSA becoming increasingly associated with invasive infections outside of the healthcare environment and MSSA becoming associated with severe HCA infections such as BSIs, endocarditis and bacteraemia (Chambers 2001; Grundmann *et al.*, 2006; David *et al.*, 2011). This is in stark contrast to a decade ago where invasive infections in the community were predominantly MSSA and HCA infections were almost always due to MRSA (David *et al.*, 2011; Miller *et al.*, 2007; Naimi *et al.*, 2003).

In Ireland, the proportion of MRSA among *S. aureus* BSIs has reduced significantly from 41.9% in 2006 to 24% in 2011 while the proportion of MSSA has increased from 58.1% in 2006 to 76% in 2011 (Table 5.1). A study by de Kraker *et al.* (2012) identified declining rates of MRSA BSIs in hospitals across Europe using the

Table 5.1 Total numbers of reported bloodstream infections among patients in Irish hospitals caused by seven EARS-Net monitored pathogens¹ between 2006 and 2013 and the proportion of these infections caused by *S. aureus* (MRSA and MSSA)²

Year	Total No. of EARS-net monitored BSIs ¹	% BSIs caused by <i>S. aureus</i> (n)	% MSSA (n)	% MRSA (n)
2006	4379	32.24% (1412)	58.1% (820)	41.9% (592)
2007	4647	29.98% (1393)	61.5% (857)	38.5% (536)
2008	4892	26.64% (1303)	66.3% (864)	33.7% (439)
2009	4986	26.25% (1309)	72.9% (954)	27.1% (355)
2010	4973	25.16% (1251)	75.6% (946)	24.4% (305)
2011	4757	23.02% (1095)	76% (832)	24% (263)
2012	5085	20.85% (1060)	77.2% (818)	22.8% (242)
2013	5213	20.99% (1094)	79.7% (872)	20.3% (222)

¹ The seven bloodstream infection pathogens monitored by the European Antimicrobial Surveillance Network (EASR-Net) are *Streptococcus pneumoniae*, *S. aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

²Data adapted from HPSC, 2014.

EARS-Net database and a steady incline among rates of MSSA BSIs between 2002 and 2008. Several other studies have also identified the increasing trend of *S. aureus* related BSIs in Europe, often characterised by a similar increase in MSSA and decrease in MRSA (Marcos *et al.*, 2011; Wilson *et al.*, 2011).

The reasons for the changing epidemiology of MRSA and MSSA causing BSIs are not well understood and several reasons for this have been proposed (Ellington *et al.*, 2010; Albrecht *et al.*, 2011; Rao *et al.*, 2011; Espadinha *et al.*, 2013) including pathogen-specific changes such as clonal decline of MRSA, or changes in MRSA/MSSA transmissibility and/or virulence or resistance potential (aires-de-Souza *et al.*, 2008; deLeo & Chambers, 2009; Holden *et al.*, 2013), and patient-specific changes including an increasing high-risk aging population and a shift in incidence between hospital-based or community-based patients (Schlebusch *et al.*, 2010; Patel *et al.*, 2013). With the large numbers of circulating MRSA and MSSA strains prevalent at any one time it is likely that strains are in competition with each other, with the fitter strains replacing less well adapted ones. Increased fitness can come about by the development or acquisition of resistance to an antibiotic newly in use in a hospital or through the acquisition of a virulence factor(s) that enable a strain to survive better or to be more pathogenic. For example, it has been speculated previously that the presence of ACME in some ST22-MRSA-IV isolates may lead to enhanced fitness in this strain by improving its ability to grow and survive on the host (Shore *et al.*, 2011).

Different HCA-MRSA strains have been shown to predominate in different geographic regions and even at different time periods (Moran *et al.*, 2006; Rasigade *et al.*, 2010; Grundmann *et al.*, 2014). For example, there have been significant changes in the predominating strains of MRSA circulating in Irish hospitals over the last 40 years (Shore *et al.*, 2005; Shore *et al.*, 2010). The ST250-MRSA-I/I-*pls* clone which predominated in the 1970's and early 1980's was replaced by ST239-MRSA-III/III-pI258/Tn554 in the 1980s. This clone was in turn displaced by ST8-MRSA-IIA-E in the 1990's and finally in 2002 ST22-MRSA-IV became the dominant clone. Despite the overall decline in MRSA BSIs in Ireland, the ST22-MRSA-IV clone continues to predominate accounting for approximately 80% of MRSA isolates from patients in Irish hospitals each year (Rossney *et al.*, 2006; Shore *et al.*, 2010).

A large European-wide study in 2008 investigated the *spa* types of *S. aureus* causing BSIs in Europe. This study characterised the dominant clones and geographic distribution of 2890 *S. aureus* isolates from BSIs throughout Europe over a six-month period in 2006/07 (Grundmann *et al.*, 2008). The study identified geographic clonal clustering among a limited number of MRSA clones and contrastingly no predominant clustering and widespread diversity among the MSSA isolates. Isolates exhibiting *spa* types associated with ST22-MRSA-IV, in particular *spa* type t032, were predominantly clustered in western Europe, including Ireland and the UK, and accounted for 16.9% of all MRSA isolates identified. In contrast, among the MSSA isolates the predominant *spa* type identified across the entire region was the ST5 *spa* type t002, which accounted for only 4.8% of isolates. In Ireland the predominant *spa* types among the MSSA isolates were the CC30 *spa* types t021 and t012 (Brennan, 2009; Deasy, 2013).

Despite the large burden and changing dynamics of BSIs attributable to both MRSA and MSSA in Ireland there has been no comprehensive analysis of *S. aureus* isolates from BSIs in Ireland since 2006 and comparatively little is known about the pathogen and patient characteristics during the decline of MRSA and increase in MSSA BSIs between 2006 and 2011. Therefore, the aims of this study were to comprehensively characterise and compare the molecular and epidemiological characteristics of MRSA and MSSA causing BSIs among patients in Irish hospitals during two different time periods when MRSA BSIs were at their peak (2006/07) and when MRSA BSIs had declined and MSSA BSIs increased (2011) in order to identify if any change in virulence factor(s), antimicrobial resistance gene(s), clonal type or patient demographics could be attributed to the decline in MRSA BSIs.

5.2 Materials and Methods

5.2.1 MRSA and MSSA isolates investigated

A total of 354 *S. aureus* isolates recovered from patients in Irish hospitals with invasive infections, mainly BSIs (352/354), were investigated. This included 83 MRSA and 84 MSSA recovered between September 30th 2006 and February 28th 2007 and 73 MRSA and 114 MSSA recovered between January 1st and June 30th 2011. All isolates were collected as part of two European *spa* typing studies from patients in Irish hospitals that participated in EARS-Net during the two study periods (Grundman *et al.*, 2010). This involved 21 hospitals from across Ireland in 2006/2007 and 25 in 2011. Up to a maximum of 10 *S. aureus* isolates were submitted by each hospital for the study, including, where possible, the first five consecutive MRSA and five consecutive MSSA. In 2011, a number of hospitals were unable to provide a total of five MRSA or five MSSA isolates; therefore the total of 10 isolates was made up by including more of the alternative, i.e. if a hospital had only three MRSA and five MSSA isolates to submit, they could add in two additional MSSAs to make the total of 10 isolates. Only one isolate per patient was included. For each isolate, patient data was also collected including gender, date of birth, the epidemiological context of the infection i.e. hospital-onset (if symptoms developed more than 48 hours after admission) or community-onset (symptoms present upon or within 48 hours of admission) (HO or CO, respectively) and all-cause mortality at 14 days after recovery of the first invasive isolate.

5.2.2 Phenotypic and genotypic analysis of isolates

All isolates were subjected to antimicrobial susceptibility testing to a panel of 23 antimicrobial agents by AR typing and were genotyped by *spa* typing and DNA microarray profiling using the StaphyType DNA microarray (to assign isolates to MLST CCs/STs and SCC*mec* types), as described in Chapter 2, sections 2.3.3, 2.4.3 and 2.4.5, respectively. The DNA microarray was also used to detect virulence and antimicrobial resistance genes among the isolates. The phenotypic and genotypic analysis of the isolates recovered in 2006/2007 was performed previously (Brennan, 2009; Deasy, 2014), whilst the analysis of the 2011 isolates and the comparison of isolates from both time periods was performed as part of the present study.

For some isolates the susceptibility phenotype to particular antimicrobial agents did not correlate with the antimicrobial resistance gene content of an isolate as identified by DNA microarray profiling. Therefore, PCRs were performed to confirm the presence or absence of the relevant genes in these isolates as described in Chapter 2 (section 2.3.3 and Table 2.1). This included PCR detection of *blaZ*, *dfrS1*, *erm(C)*, *qacA*, *qacC* and *tet(K)*.

5.2.3 Comparison of patient and isolate data

The comparative analysis of all data was performed using the Microsoft Excel software package (version 14.0). Patient data, *spa* types, CCs/STs and SCC*mec* types, antimicrobial susceptibility patterns and virulence and antimicrobial resistance gene carriage were compared for MRSA and MSSA isolates across the two study periods. Statistical analysis was performed using STATA Version 13.0 (StatCorp, Texas, USA). Where STATA identified significant differences between the two study periods the individual differences in the proportions between two years were tested using the Z-test for Two Proportions for numerical data, or the Chi Square tests for categorical data. Results were considered significant if the tests resulted in a *p*-value <0.05, at a 95% confidence level.

5.3 Results

5.3.1 Genotyping

Seven MLST-SCC*mec* genotype combinations were identified among the 83 MRSA isolates recovered in 2006/07 and nine among the 73 MRSA isolates recovered in 2011 (Fig. 5.1a). The predominant MRSA genotype during both study periods was CC/ST22-MRSA-IV accounting for 85.5% (71/83) of MRSA in 2006 and 87.7% (64/73) in 2011. Only two other genotypes were represented by isolates from both study periods but these genotypes were represented by a small number of isolates (Fig. 5.1a) i.e. CC/ST8-MRSA-II accounted for 6% (5/83) and 2.7% (2/73) of MRSA in 2006/07 and 2011, respectively, and ST5-MRSA-II accounted for 3.6% (3/83) and 1.4% (1/73) of MRSA in 2006/07 and 2011, respectively. The remaining MRSA genotypes (four in 2006/07 and six in 2011) were unique to the specific study periods and accounted for one isolate each (Fig. 5.1a)

A total of 36 *spa* types were identified among the MRSA isolates, 25 in 2006/07 and 21 in 2011 with the CC/ST22-MRSA-IV *spa* type t032 predominating and accounting for almost half of the isolates investigated during both study periods (2006/07: 45.8%, 38/83; 2011: 45.2%, 33/73) (Table 5.2). Ten *spa* types were common among MRSA isolates from both study periods, the majority of which were CC/ST22-MRSA-IV-related *spa* types (t022, t025, t032, t1214, t1292, t2945, t515, t578 and t747) with one belonging to CC/ST8-MRSA-II (t190) (Table 5.2). All common *spa* types were represented by similar number of isolates ($n \leq 4$) in both study periods and any differences identified were not statistically significant (Table 5.2). A total of 15 *spa* types were unique to MRSA isolates in 2006/07 and included *spa* types within CC5, CC8, CC22, CC45 and CC8/ST239 while 10 were unique in 2011 and included CC1, CC30 and CC59 *spa* types (Table 5.2). All unique *spa* types were represented by three isolates or less.

Sixteen and 21 MLST genotypes (CCs/STs) were identified among 84 MSSA isolates in 2006/07 and 114 MSSA isolates in 2011, respectively (Fig. 5.1b). Overall, 13 genotypes were common to MSSA isolates during both study periods and the dominant MSSA clones identified during both study periods were CC30, CC45 and CC5 (Fig. 5.2b). However, the proportions of these three clones varied during the two study periods (Fig. 5.1b). In 2006/07 CC30-MSSA accounted for 23.8% (20/84) of all MSSA isolates identified but this declined significantly to 10.5% (12/114) in 2011 ($p = 0.012$). In

2006/07, CC45-MSSA was the second most common MSSA clone accounting for 17.9% (15/84) of MSSA isolates and although it accounted for a similar proportion of MSSA isolates in 2011 (15.8%, 18/114) it was the most common MSSA clone identified in 2011. The CC5-MSSA clone accounted for similar proportions of isolates during both study periods (2006/07: 11.9%, 10/84; 2011: 11.4%, 13/114) but it was the third most common clone in 2006/07 and the second most common clone in 2011. Four and nine MSSA genotypes were unique to isolates recovered in 2006/07 and 2011, respectively, but isolate numbers within each of these were low (<5 isolates each, Fig. 5.1.b).

Among the MSSA isolates 105 *spa* types were identified, 54 in 2006/07 and 74 in 2011 (Table 5.2). Overall, the majority of *spa* types (90/103 *spa* types) were represented by one to three isolates only. The remaining MSSA *spa* types were represented by four to 10 isolates each (CC45 t065 & t230, CC5 t088, t002 & t548, CC34 t089, CC8 t008, CC15, t084, CC34 t166, CC30 t012 & t021, CC7 t091 and CC1 t127) (Table 5.2).

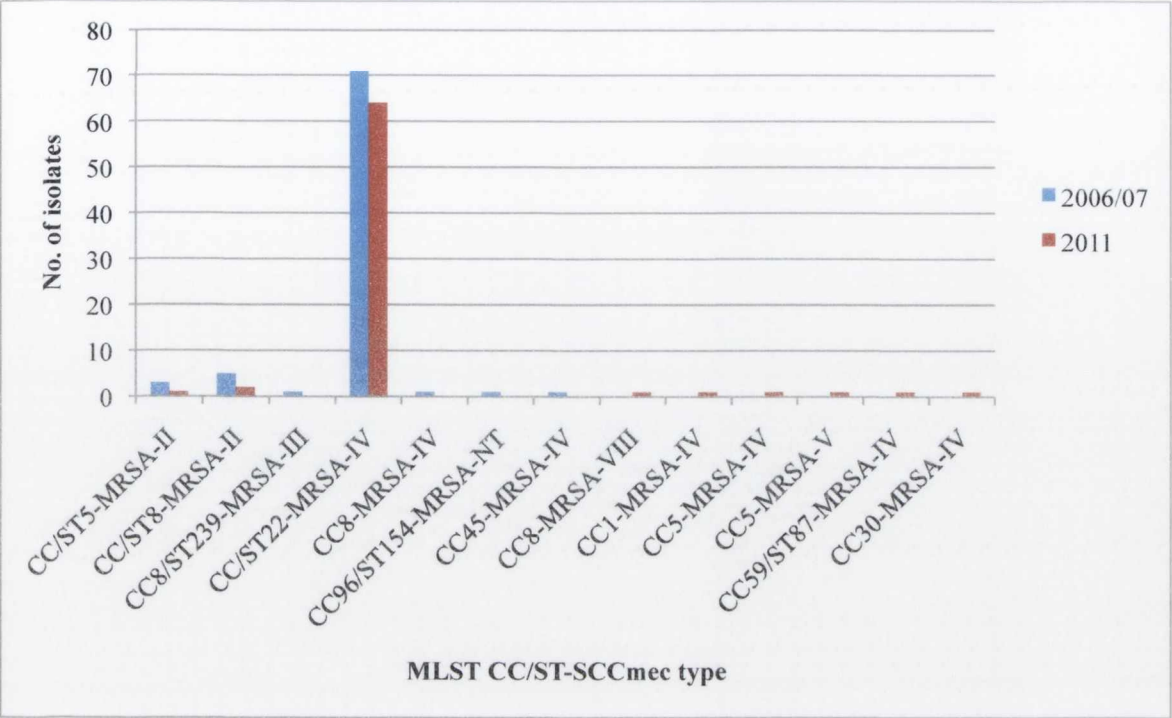
Twenty-two *spa* types were common to MSSA during both study periods and included *spa* types within CC1, CC5, CC7, CC8, CC9, CC12, CC15, CC22, CC25/28, CC30-ST34, CC45 and CC121 (Table 5.2). A total of 31 *spa* types were unique to the 2006/07 study period and 52 were uniquely identified in 2011. However, similar to the MRSA isolates, all *spa* types uniquely identified in any given study period were represented by one to three isolates only (Table 5.2).

There were no *spa* types common to both MRSA and MSSA in 2006/07 but five common *spa* types represented by 40 MRSA and 17 MSSA isolates were identified in 2011 and included CC5 t002 (three MRSA & five MSSA), CC22 t005 (two MRSA & one MSSA), CC30 t012 (one MRSA & three MSSA), CC22 t032 (33 MRSA & one MSSA) and CC1 t127 (one MRSA & seven MSSA) (Table 5.2).

5.3.2 Antimicrobial resistance

Similar antimicrobial resistance phenotypes and rates of carriage of antimicrobial resistance genes were detected among MRSA isolates from both study periods (Fig. 5.2a and Table 5.3). Apart from cefoxitin resistance encoded by *mecA* (detected in 100% of MRSA isolates), the most common resistances detected were beta-lactam resistance encoded by *blaZ* (2006/07: 100%, 83/83; 2011: 98.6%, 72/84) and resistance to

(a)



(b)

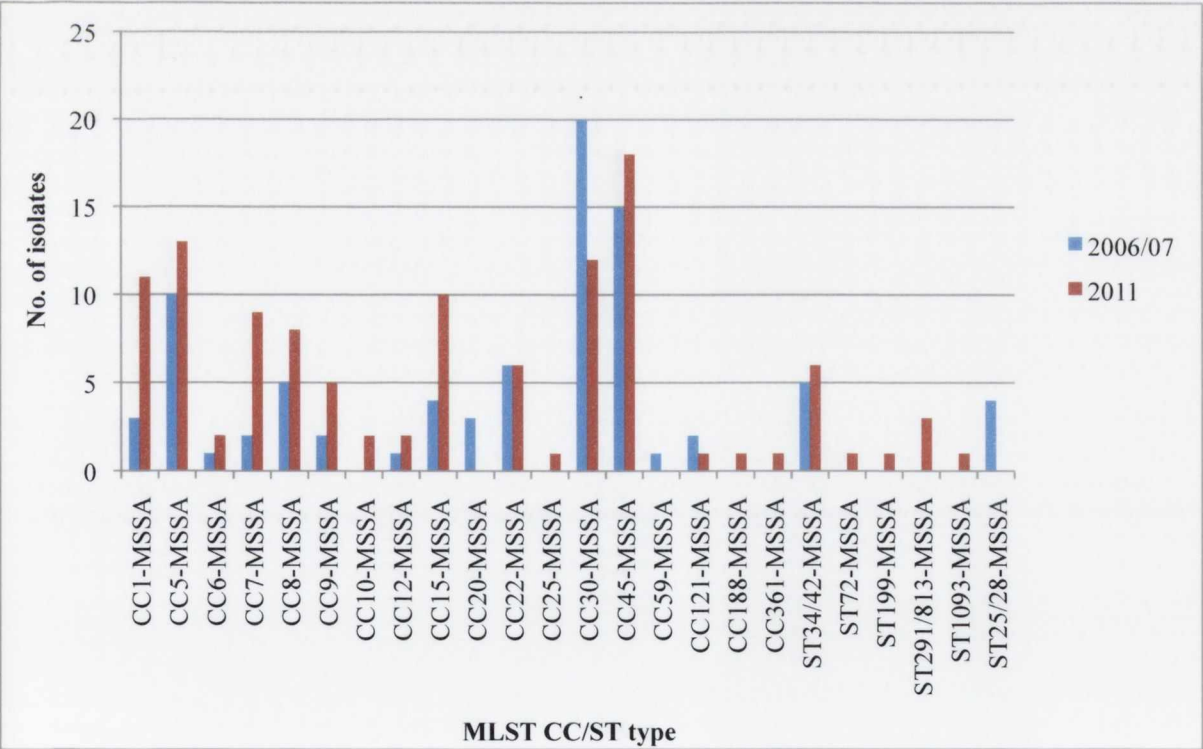
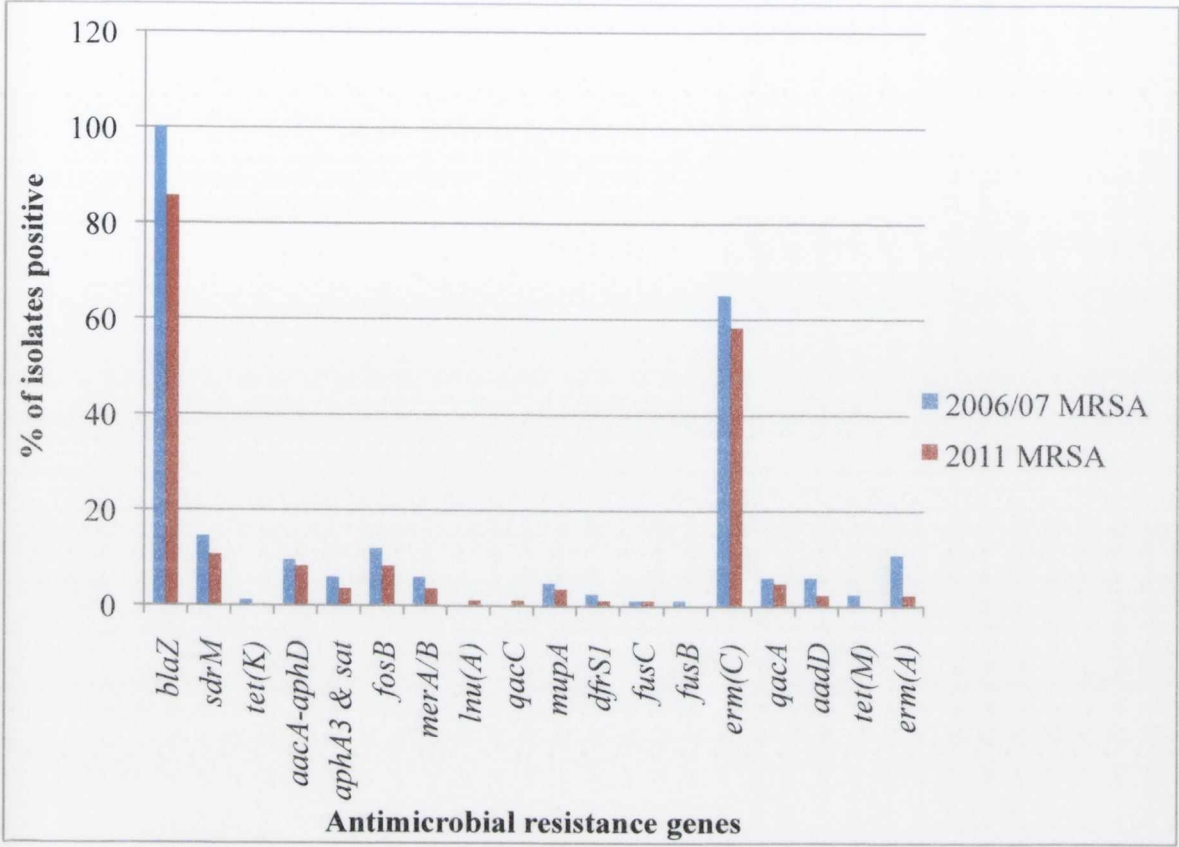


Figure 5.1 MLST sequence types (STs) and/or clonal complexes (CCs) and SCCmec types (for MRSA only) identified among (a) MRSA isolates and (b) MSSA isolates recovered from patients with bloodstream infections in Irish hospitals during two six-month periods in 2006/07 and 2011. NT, not typeable SCCmec with class C mec complex, *ccrAA* and *ccrB4*. Statistically significant differences are indicated with an asterisk (*).

(a)



(b)

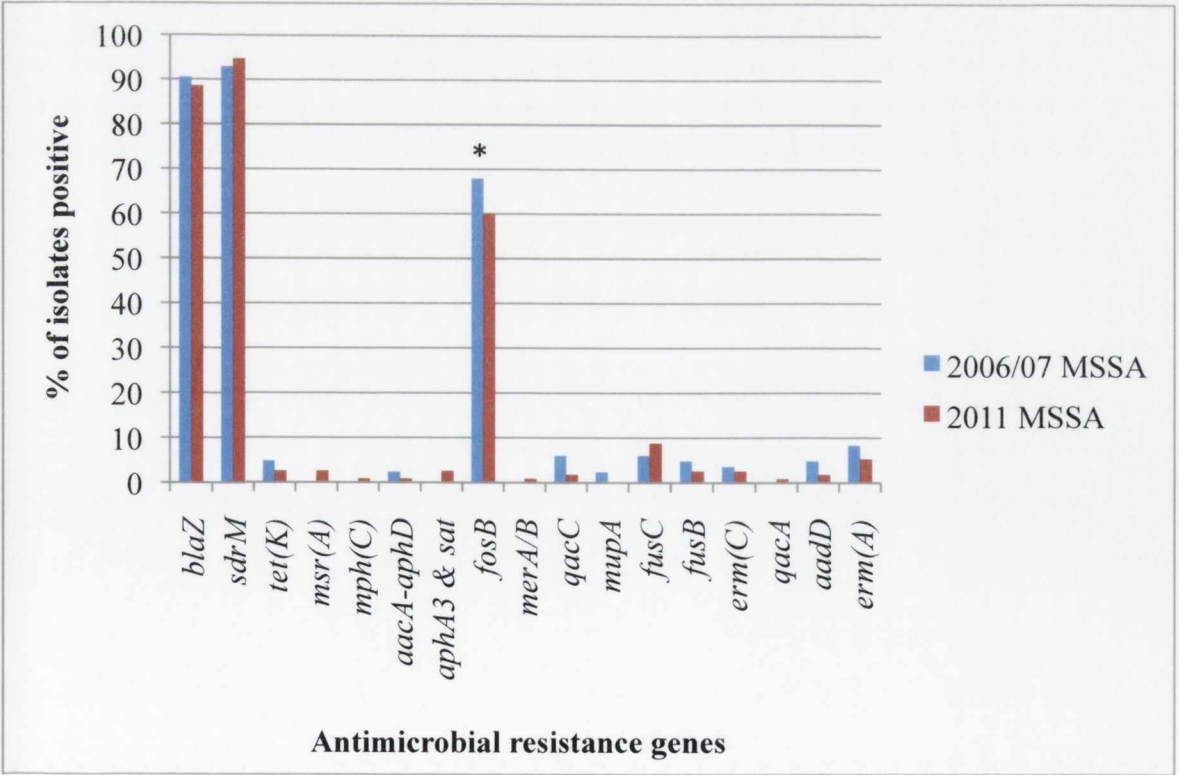


Figure 5.2 Antimicrobial resistance genes detected using the StaphyType DNA microarray (Alere) among (a) MRSA and (b) MSSA isolates recovered from patients with bloodstream

infections in Irish hospitals during two six-month periods in 2006/07 and 2011. Only genes for which the isolates were positive are shown, apart from *mecA*, which is not shown but was harboured by all MRSA isolates and by none of the MSSA isolates. Table 2.4 shows a full list of antimicrobial resistance genes that can be detected by the DNA microarray and what they encode resistance to. Statistically significant differences are indicated with an asterisk (*).

Table 5.2. *spa* types identified among isolates of each genotype of MRSA and MSSA recovered from patients with bloodstream infections in Irish hospitals during two study periods in 2006/07 and 2011

Genotype ¹	<i>spa</i> type	No. of MRSA isolates ²		No. of MSSA isolates ²	
		2006/07	2011	2006/07	2011
CC1-MRSA-IV CC1-MSSA	t127	-	1	-	-
	t098	-	-	-	1
	t127	-	-	3	7
	t174	-	-	-	1
	t1491	-	-	-	1
	t10094	-	-	-	1
CC/ST5-MRSA-II	t045	2	-	-	-
	t463	1	-	-	-
	t002	-	1	-	-
CC5-MRSA-IV	t002	-	1	-	-
CC5-MRSA-V	t002	-	1	-	-
CC5-MSSA	t002	-	-	2	5
	t071	-	-	1	-
	t088	-	-	3	1
	t179	-	-	1	-
	t548	-	-	3	5
	t769	-	-	-	1
	t10989	-	-	-	1
CC6-MSSA	t701	-	-	-	2
	t2360	-	-	1	-
CC7-MSSA	t091	-	-	2	7
	t289	-	-	-	1
	t9565	-	-	-	1
CC/ST8-MRSA-II	t190	4	2	-	-
	t2942	1	-	-	-
CC8-MRSA-IV	t064	1	-	-	-
CC8-MRSA-VIII	t190	-	1	-	-
CC8-MSSA	t008	-	-	2	3
	t024	-	-	1	1
	t211	-	-	-	1
	t334	-	-	-	1
	t1391	-	-	1	-
	t1852	-	-	-	1
	t2658	-	-	1	-
	t10992	-	-	-	1
CC8/ST72-MSSA	t148	-	-	-	1
CC8/ST239-MRSA-III	t030	1	-	-	-

Continued Overleaf

Genotype ¹	<i>spa</i> type	No. of MRSA isolates ²		No. of MSSA isolates ²	
		2006/07	2011	2006/07	2011
CC9-MSSA	t100	-	-	-	1
	t209	-	-	2	1
	t1045	-	-	-	1
	t3098	-	-	-	1
	t10259	-	-	-	1
CC10-MSSA	t10097	-	-	-	1
	t647	-	-	-	1
CC12-MSSA	t156	-	-	-	1
	t160	-	-	1	1
C15-MSSA	t084	-	-	2	3
	t228	-	-	-	3
	t279	-	-	1	-
	t346	-	-	-	1
	t803	-	-	-	1
	t1716	-	-	-	1
	t2574	-	-	-	1
	t2949	-	-	1	-
CC15/ST199- MSSA	t279	-	-	-	1
CC20-MSSA	t2094	-	-	1	-
	t2943	-	-	1	-
	t2947	-	-	1	-
CC/ST22-MRSA- IV	t005	-	2	-	-
	t020	2	-	-	-
	t022	4	2	-	-
	t025	3	2	-	-
	t032	38	33	-	-
	t379	-	3	-	-
	t515	8	7	-	-
	t578	1	2	-	-
	t651	-	1	-	-
	t718	1	-	-	-
	t747	1	1	-	-
	t785	1	-	-	-
	t885	2	-	-	-
	t1214	2	1	-	-
	t1292	1	1	-	-
	t1802	2	-	-	-
	t1865	1	-	-	-
	t2231	1	-	-	-
	t2945	2	4	-	-
	t2951	1	-	-	-

Continued Overleaf

Genotype ¹	<i>spa</i> type	No. of MRSA isolates ²		No. of MSSA isolates ²	
		2006/07	2011	2006/07	2011
CC22-MSSA	t4102	-	1	-	-
	t4765	-	1	-	-
	t7983	-	2	-	-
	t10096	-	1	-	-
	t005	-	-	2	1
	t032	-	-	-	1
	t223	-	-	1	2
	t449	-	-	1	-
	t790	-	-	-	1
	t2787	-	-	1	-
	t2938	-	-	1	-
	t4565	-	-	-	1
CC25/28-MSSA	t078	-	-	2	1
	t349	-	-	1	-
	t2939	-	-	1	-
CC30-MRSA	t012	-	1	-	-
CC30-MSSA	t012	-	-	4	3
	t018	-	-	1	-
	t021	-	-	6	2
	t342	-	-	-	1
	t338	-	-	1	1
	t382	-	-	3	-
	t399	-	-	1	-
	t1414	-	-	1	-
	t1793	-	-	-	1
	t1827	-	-	1	-
	t2944	-	-	1	-
	t2950	-	-	1	-
	t4341	-	-	-	1
	t5866	-	-	-	1
	t8304	-	-	-	1
	t10991	-	-	-	1
CC30/ST34-MSSA	t089	-	-	2	2
	t166	-	-	3	3
	t5337	-	-	-	1
CC45-MRSA-IV	t727	1	-	-	-
CC45-MSSA	t015	-	-	1	1
	t026	-	-	2	-
	t050	-	-	-	1
	t065	-	-	2	2
	t230	-	-	2	5
	t331	-	-	1	-
	t362	-	-	1	-
	t383	-	-	-	1
	t620	-	-	-	1
	t715	-	-	-	1

Continued Overleaf

Genotype ¹	<i>spa</i> type	No. of MRSA isolates ²		No. of MSSA isolates ²	
		2006/07	2011	2006/07	2011
	t1040	-	-	-	1
	t1321	-	-	1	-
	t2143	-	-	1	1
	t2371	-	-	-	1
	t2598	-	-	-	1
	t2940	-	-	1	-
	t2941	-	-	1	-
	t2946	-	-	1	-
	t2948	-	-	1	-
	t5529	-	-	-	1
	t10990	-	-	-	1
CC59-MRSA-IV	t316	-	1	-	-
CC59-MSSA	t216	-	-	1	-
CC96/154-MRSA	t878	1	-	-	-
CC121-MSSA	t171	-	-	2	1
CC188-MSSA	t189	-	-	-	1
CC398/ST291/813- MSSA	t1614	-	-	-	1
	t3096	-	-	-	2
CC361-MSSA	t1309	-	-	-	1
ST1093-MSSA	t4842	-	-	-	1

¹ Genotypes were determined by DNA microarray profiling where isolates were assigned to MLST clonal complexes (CCs)/sequence types (STs) and, for MRSA, SCC*mec* types.

² -, no isolates representative of this *spa* type were identified among the isolates indicated.

Table 5.3 Antimicrobial resistance phenotypes, resistance genes and virulence genes detected among the different genotypes of MRSA identified from patients with bloodstream infections in Irish hospitals in 2006/07 and 2011

Genotype	n	Antimicrobial resistance (n) ^{1,2}		Antimicrobial resistance genes (n) ²		Virulence genes (n) ^{2,3}	
		2006/07	2011	2006/07	2011	2006/07	2011
CC1-MRSA-IV	0	1	N/A ⁴	Ap, Cd, Er, Fd	<i>blaZ</i> , <i>sdrM</i> , <i>fusC</i> , <i>erm</i> (C)	N/A	<i>sea</i> , <i>sek/q</i> , <i>seh</i> , IEC-G, <i>icaA/C/D</i> ,
CC/ST5-MRSA-II	3	1	Ap, Cd (1), Cp (2), Er, Fd (2), Kn (2), Ln (2), Nm (2), Sp, Tb (2)	Ap, Cp, Er, Ln, Sp	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>aadD</i> (2)	<i>sea</i> , <i>sed/j/r</i> , <i>tst</i> , <i>egc</i> , IEC-A(1), IEC-B(1), IEC-D(1), <i>icaA/C/D</i>	<i>sed/j/r</i> , <i>egc</i> , , <i>icaA/C/D</i>
CC/ST5-MRSA-IV	0	1	N/A	Ap, Cd, Eb, Er, Mc, Pma	<i>blaZ</i> , <i>sdrM</i> , <i>aphA3&sat</i> , <i>fosB</i> , <i>merA/B</i> , <i>erm</i> (C), <i>qacA</i> , <i>sdrM</i> , <i>aacA</i> <i>aphD</i> , <i>apphA3&sat</i> , <i>fosB</i>	N/A	<i>sea</i> , <i>egc</i> , IEC-F, <i>icaA/C/D</i>
CC/ST5-MRSA-V	0	1	N/A	Ap, Cd, Gn, Kn, Tb	N/A	N/A	<i>sea</i> , <i>sed/j/r</i> , <i>egc</i> , IEC-G, <i>icaA/C/D</i>
CC/ST8-MRSA-II	5	2	Ap, Cd, Cp (3), Eb (3), Er, Gn, Kn, Ln (2), Mc (3), Nm, Pma (3), Sm, Sp, Tb	Ak (1), Ap, Cd, Cp, Eb, Er, Gn, Kn, Mc (1), Mp, Nm, Pma (1), Sp, St (1), Tb, Tp (2), <i>erm</i> (A)	<i>blaZ</i> , <i>sdrM</i> , <i>aacA</i> <i>aphD</i> , <i>aphA3&sat</i> , <i>fosB</i> , <i>merA/B</i> (5), <i>mupA</i> (1), <i>qacA</i> , <i>aadD</i> (1)	<i>sea</i> , IEC-D, D(3), <i>sea/sak</i> (2), <i>icaA/C/D</i>	<i>sea</i> , IEC-D, <i>icaA/C/D</i>

Genotype	<i>n</i>	Antimicrobial resistance (<i>n</i>) ^{1,2}		Antimicrobial resistance genes (<i>n</i>) ²		Virulence genes (<i>n</i>) ^{2,3}	
	2006/07	2011	2006/07	2011	2006/07	2011	2006/07
CC8-MRSA-IV	1	0	Ap, Cp, Er, Gn, Kn, Ln, Nm, Su, Tb, Te, Tp	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>aacA_aphD</i> , <i>fosB</i> , <i>dfrSI</i> , <i>aadD</i> , <i>tet(L)</i>	N/A	<i>sea</i> , <i>seb</i> , <i>sek/q</i> , IEC-D, <i>icaA/C/D</i>
CC8-MRSA-VIII	0	1	N/A	AP, Cd, Cp, Eb, Er, Gn, Kn, Mc, Mp, Nm, Pma, Sp, St, Tb, Tp	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>aacA_aphD</i> , <i>aphA3&sat</i> , <i>fosB</i> , <i>qacA</i>	N/A
CC/ST22-MRSA-IV	71	64	Ap, Cd (26), Cl (1), Cp (66), Er (53), Fd (20), Gn (1), Kn (1), Ln (1), Mp (3), Rf (1), Tb (1), Te (1), Tp (1)	AP, Cd, Cp, Eb, Er, Gn, Kn, Mc, Mp, Nm, Pma, Sp, St, Tb, Tp	<i>blaZ</i> , <i>tet(K)</i> (1), <i>aacA_aphD</i> (1), <i>mupA</i> (3), <i>dfrSI</i> (1), <i>erm(C)</i> (53)	<i>blaZ</i> , <i>aacA-aphD</i> (3), <i>lnuA</i> (1), <i>qacC</i> (1), <i>mupA</i> (3), <i>dfrSI</i> (1), <i>erm(C)</i> (46)	<i>seb</i> (1), <i>sec/I</i> (25), ACME- <i>arc</i> (1), <i>egc</i> , IEC-B (68), <i>icaA/C</i> (4 <i>amb</i> ⁴)/ <i>D</i> (52)
CC30-MRSA	0	1	N/A	Te (1), Tp (1) Ak, Ap, Cd, Cp, Er, Kn, Ln, Mp, Nm, Sp, Tb	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>aadD</i> , <i>erm(A)</i>	N/A
CC45-MRSA-IV	1	0	Ap, Cd, Cp, Er, Fd	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>merA/B</i> , <i>fusB</i> , <i>erm(C)</i>	N/A	<i>egc</i> , IEC-B, <i>icaA/C/D</i>
CC59-MRSA	0	1	N/A	Ap	N/A	<i>blaZ</i> , <i>sdrM</i>	N/A
							<i>sea</i> , <i>tst</i> , <i>egc</i> , IEC-A, <i>icaA/C/D</i>
							<i>seb</i> , <i>sek/q</i> , IEC-C, <i>icaA/C/D</i>

Continued Overleaf

Genotype	n	Antimicrobial resistance (n) ^{1,2}		Antimicrobial resistance genes (n) ²		Virulence genes (n) ^{2,3}	
	2006/07	2011	2006/07	2011	2006/07	2011	2011
CC96/154-MRSA	1	0	Ap, Fd	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>fus(C)</i>	N/A	<i>seb</i> , <i>etD&edinB</i> , IEC-B, <i>icaA/C/D</i>
ST239-MRSA-III	1	0	Ak, Ap, Cd, Cl, Cp, Er, Gn, Kn, Rf, Sm, Sp, Tb, Te	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>aacA_aphD</i> , <i>fosB</i> , <i>tet(K)</i> , <i>erm(A)</i>	N/A	<i>sea</i> , <i>sek/q</i> , IEC-D, <i>icaA/C/D</i>

¹ Susceptibility of isolates was determined to 23 antimicrobial agents including amikacin (Ak), ampicillin (Ap), cadmium acetate (Cd), chloramphenicol (Cl), ciprofloxacin (Cp), ethidium bromide (Eb), erythromycin (Er), fusidic acid (Fd), gentamicin (Gn), kanamycin (Kn), lincomycin (Ln), mercuric chloride (Mc), mupirocin (Mp), neomycin (Nm), phenyl mercuric acetate (Pma), rifampicin (Rf), sulphonamide (Su), spectinomycin (Sp), streptomycin (St), tobramycin (Tb), tetracycline (Te), trimethoprim (Tp), vancomycin.

² The number of isolates are indicated when not all isolates within a given genotype exhibited the same characteristic.

³ Immune evasion cluster (IEC) types were determined as described previously by Van Wamel *et al.*, (2008). A: *sea*, *sak*, *chp* & *scn*; B: *sak*, *chp* & *scn*; C: *chp* & *scn*; D: *sea*, *sak* & *scn*; E: *sak* & *scn*; F: *sep*, *sak*, *chp* & *scn*; G: *sep*, *sak* & *scn*.

⁴ N/A not applicable, no isolates of this genotype identified during the study period indicated

⁵ AMB, the number of isolates yielding an ambiguous signal for *icaC* using the DNA microarray are indicated.

macrolides, lincosamides and streptogramin B compounds (MLS_B) encoded by *erm*(C) (2006/07: 65.1%, 54/83; 2011: 67.1% 49/84) (Fig. 5.2a).

Resistance to ciprofloxacin, fusidic acid and cadmium were also common among the MRSA isolates (Table 5.3). Ciprofloxacin resistance was detected among 91% of MRSA isolates (142/156) with no significance difference between the MRSA isolates in the two study periods (2006/07: 89.2%, 74/83; 2011: 93.2%, 68/73). Ciprofloxacin resistance is commonly encoded by mutations in *GyrA* and is not detected on the DNA microarray. Fusidic acid resistance was also common among the MRSA isolates and there was no significant difference between both study periods (2006/07: 28.9%, 24/83; 2011: 32.9%, 24/73). For 8.3% (4/48) of isolates exhibiting resistance to fusidic acid relevant genes were detected including *fusB* (2006/07: 4.2%, 1/24; 2011: 0%, 0/24) and *fusC* (2006/07: 4.2%, 1/24; 2011: 8.3%, 2/24). However, for the majority of fusidic acid resistant isolates (91.7%, 44/48), which were predominantly CC/ST22-MRSA-IV, no relevant resistance genes were detected. A previous study from this laboratory has shown fusidic acid resistance in this clone to be predominantly encoded by mutations in *fusA*, which is not detected using the DNA microarray (Brennan, 2013). Resistance to cadmium was also common among MRSA from both study periods (2006/07: 41%, 34/83; 2011: 49.3%, 36/73) but cadmium resistance genes (*cadA-D*) are not detected using the DNA microarray.

All other antimicrobial resistance genes and phenotypes detected among MRSA isolates during both study periods were each detected among a small number of isolates only and at similar levels during both study periods (Fig. 5.2a and Table 5.3). Five antimicrobial resistance genes were unique to MRSA detected during either study period, three in 2006/07 i.e. the tetracycline resistance genes *tet*(K) and *tet*(M) and the fusidic acid resistance gene *fusB*, and two in 2011, the lincosamide resistance gene *lnu*(A) and quaternary ammonium compound (QAC) resistance gene *qacC*, but each was detected in one or two isolates only (Fig. 5.2a).

Within the most common MRSA clone identified, CC/ST22-MRSA-IV, there was no significant difference in the prevalence of antimicrobial resistance genes or phenotype among isolates during both study periods (Table 5.3). Overall, the CC/ST22-MRSA-IV isolates harboured eight antimicrobial resistance genes and exhibited resistance to 19 of the 23 antimicrobial agents tested (Table 5.3).

The most common antimicrobial resistance gene detected among the MSSA was the multidrug efflux pump gene *sdrM* (2006/07: 92.9%, 78/84; 2011: 94.7%, 108/114) followed closely by beta-lactam resistance gene *blaZ* (2006/07: 90.5%, 76/84; 2011: 88.6%, 101/114) (Fig. 5.2b). The fosfomycin resistance gene *fosB* was also common but its prevalence declined significantly from 67.9% (57/84) in 2006/07 to 52.6% (60/114) in 2011 ($p = 0.031$) (Fig. 5.2b). The *fosB* gene was detected among MSSA isolates belonging to 15 genotypes and there was a decline in the total numbers of isolates from five of these genotypes (CC20: 2006/07, 3; 2011, 0; CC25: 2006/07, 4; 2011, 1; CC30: 2006/07, 20; 2011, 12; ST34: 2006/07, 5; 2011, 0; CC121: 2006/07, 2; 2011, 1) between the two study periods which accounted for the decrease in *fosB* prevalence (Table 5.4). There were no other significant differences in the rates of carriage of antimicrobial resistance genes among the MSSA isolates during the two study periods or between MSSA belonging to the same genotype between both study periods (Fig. 5.2b). Among the MSSA, one antimicrobial resistance gene, *mupA* encoding high-level mupirocin resistance, was only detected among isolates in 2006/07, and five antimicrobial resistance genes were unique to 2011 i.e. the macrolide resistance gene *msr(A)* & *mph(C)*, the aminoglycoside and streptothricin resistance gene cluster *aphA3* & *sat*, the mercury resistance genes *merA* & *merB* and the QAC resistance gene *qacA*. However, in each instance these genes were represented by less than three MSSA isolates each (Fig 5.2b).

Three antimicrobial resistance genes, *lnuA*, *dfrS1* and *tet(M)*, were detected among the MRSA isolates but not among MSSA isolates in the present study. Conversely, two antimicrobial resistance genes, *msr(A)* and *mph(C)*, were unique to the MSSA isolates. Again, the numbers of isolates positive for the unique antimicrobial resistance genes were low and the differences were not statistically significant (Fig. 5.2b). However, a significant difference was detected in the rates of carriage of *erm(C)*, *fosB* and *sdrM* between MRSA and MSSA with *erm(C)* being more common among MRSA (MRSA: 66%, 103/156; MSSA: 3%, 6/198; $p = 0$), and *fosB* (MRSA: 10.9%, 17/156; MSSA: 59.1%, 117/198; $p = 0$) and *sdrM* (MRSA: 13.5%, 21/156; MSSA: 93.9%, 186/198; $p = 0$) being more prevalent among the MSSA (Fig. 5.2a and b).

Among the MSSA CCs 5, 8, 9 and 15 carried the most antimicrobial resistance genes and exhibited resistance to the most antimicrobial agents (Table 5.4). In contrast to the MRSA, CC22 MSSA were much less resistant with only *blaZ* and resistance to only four of the 23 antimicrobial agents tested being identified.

Table 5.4 Antimicrobial resistance phenotype, resistance genes and virulence genes detected among the different genotypes of MSSA identified from patients with bloodstream infections in Irish hospitals in 2006/07 and 2011

Genotype	<i>n</i>	Antimicrobial resistance ¹ (<i>n</i>) ²		Antimicrobial resistance genes (<i>n</i>) ²		Virulence genes (<i>n</i>) ²	
	2006/07	2011	2006/07	2011	2006/07	2011	2006/07
MSSA							
CC1-MSSA	3	11	Ap (2), Er (1), Fd (2), Sp (1)	Ap (10), Cd(I) (8), Fd (7), Tp (1)	<i>blaZ</i> (2), <i>sdrM</i> , <i>fus</i> (C) (2), <i>erm</i> (A) (1)	<i>blaZ</i> (10), <i>sdrM</i> , <i>fus</i> (C) (7)	<i>sea</i> , <i>seb</i> (1), <i>sek/q</i> , <i>seh</i> , IEC-D ³ , <i>icaA/C/D</i>
							<i>sea</i> (7), <i>sec/l</i> (1), <i>sek/q</i> (6), <i>seh</i> , <i>tst</i> (2), IEC-A(1), B(1), D(6), E(1), <i>icaA/C/D</i>
CC5-MSSA	10	13	Ap (8), Cp (1), Er (2), Fd (2), Kn (1), Ln (1), Nm (1), Sp (1), Su (1), Tb (1)	Ap (9), Cd (4), Er (3), Fd (1)	<i>blaZ</i> (9), <i>sdrM</i> , <i>fosB</i> , <i>erm</i> (C) (2), <i>aadD</i> (1), <i>erm</i> (A) (1)	<i>blaZ</i> (10), <i>sdrM</i> , <i>fosB</i> , <i>fus</i> (B) (1), <i>erm</i> (C) (2), <i>qacA</i> (1), <i>erm</i> (A) (1)	<i>sea</i> (7), <i>sed/j/r</i> (7), <i>egc</i> , IEC-B(1), D(3), F(4), <i>icaA/C/D</i>
							<i>sea</i> (8), <i>sed/j/r</i> (2), <i>tst</i> (1), <i>egc</i> , IEC-B(1), D(1), E(1), F(7), <i>etA</i> (1), <i>icaA/C/D</i>
CC6-MSSA	1	2	Ap	Ap, Cd(I)	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>	<i>sea</i> , IEC-D, <i>icaA/C/D</i>
CC7-MSSA	2	9	Ap, Ln (1), Nm (1), Tb (1), Te (1)	Ap, Cd(I) (8), Cp(I) (1), Er (1), Sp (1)	<i>blaZ</i> , <i>sdrM</i> , <i>tet</i> (K) (1), <i>aadD</i> (1)	<i>blaZ</i> , <i>sdrM</i> , <i>erm</i> (A) (1)	<i>sea</i> , IEC-G, <i>icaA/C/D</i>
CC8-MSSA	5	8	Ak (1), Ap, Cp (1), Er (1), Fd (3), Gn (2), Kn (2), Ln (2), Mp (1), Nm (1), Su (1), Tb (2), Te (1)	Ap, Cd(I) (4), Er (1), Fd (3), Ln (1), Te (1)	<i>blaZ</i> , <i>sdrM</i> , <i>tet</i> (K) (1), <i>aacA_aphD</i> (2), <i>fosB</i> , <i>qacC</i> (2), <i>mupA</i> (1), <i>fus</i> (C) (3), <i>erm</i> (C) (1), <i>aadD</i> (1)	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>qacC</i> (1), <i>fus</i> (C) (3), <i>erm</i> (C) (1)	<i>sea</i> (5), <i>sek/q</i> (2), <i>sed/j/r</i> (2), <i>tst</i> (1), <i>egc</i> (1), IEC-B(1), D(4), E(2), G(1), <i>icaA/C/D</i>

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Genotype	<i>n</i>	Antimicrobial resistance ¹ (<i>n</i>) ²		Antimicrobial resistance genes (<i>n</i>) ²		Virulence genes (<i>n</i>) ²	
		2006/ 07	2011	2006/07	2011	2006/07	2011
CC9-MSSA	2	5	Ap, Cd (1), Er	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>erm</i> (A)	<i>blaZ</i> , <i>sdrM</i> , <i>msrA</i> (1), <i>fosB</i> , <i>merA/B</i> (1), <i>erm</i> (A) (1) <i>blaZ</i> (1), <i>sdrM</i>	egc, IEC-B, <i>etA</i> , <i>icaA/C/D</i>	<i>seb</i> (1), egc, IEC- B, <i>etA</i> (1), <i>icaA/C/D</i>
CC10-MSSA	0	2	N/A ⁴	N/A		N/A	<i>seb</i> (1), <i>seh</i> (1), egc, IEC-B(1), E(1), <i>icaA/C/D</i> <i>sea</i> , <i>seb</i> (1), <i>sec/I</i> , IEC-G, <i>icaA/C/D</i> <i>sea</i> (1), <i>lukF/S-PV</i> (1), IEC-C(8), <i>sea/chp/scn</i> (1), <i>icaA/C/D</i> N/A
CC12-MSSA	1	2	None	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>	<i>sdrM</i> , <i>msrA</i> (1), <i>fosB</i>	<i>sea</i> , IEC-G, <i>icaA/C/D</i>	<i>sea</i> , <i>seb</i> (1), <i>sec/I</i> , IEC-G, <i>icaA/C/D</i> <i>sea</i> (1), <i>lukF/S-PV</i> (1), IEC-C(8), <i>sea/chp/scn</i> (1), <i>icaA/C/D</i> N/A
CC15-MSSA	4	10	Ap (3)	<i>blaZ</i> (3), <i>sdrM</i> , <i>tet</i> (K) (1), <i>fosB</i>	<i>blaZ</i> (8), <i>sdrM</i> , <i>tet</i> (K) (2), <i>fosB</i> , <i>qacC</i> (1), <i>aadD</i> (1)	IEC-C, <i>icaA/C/D</i>	
CC20-MSSA	3	0	Ap (2), Te (1)	<i>blaZ</i> , <i>sdrM</i> , <i>tet</i> (K) (1), <i>fosB</i>	N/A	egc, IEC-B, <i>etA</i> , <i>icaA/C/D</i>	<i>sec/I</i> (2), egc, IEC- B, <i>icaA/C</i> (4)/D
CC22-MSSA	6	6	Ap, Cd (3)	<i>blaZ</i>	<i>blaZ</i>	<i>sec/I</i> (1), egc, IEC- B, <i>icaA/C/D</i>	
CC25/28- MSSA	4	1	Ap, Cd (1), Su (1), Tp (1)	<i>blaZ</i> (3), <i>sdrM</i> , <i>fosB</i>	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>	<i>seb</i> (2), <i>sec/I</i> (1), <i>lukF/S-PV</i> (1), <i>etD&edinB</i> , egc, IEC-B, <i>etB</i> (1), <i>icaA/C/D</i>	<i>seb</i> , <i>etD&edinB</i> , egc, IEC-B, <i>icaA/C/D</i>
CC30-MSSA	20	12	Ap (4), Cd (7), Er (1), Sp (1)	<i>blaZ</i> (18), <i>sdrM</i> , <i>fosB</i> , <i>qacC</i> (2), <i>erm</i> (A) (1)	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>erm</i> (A) (1)	<i>sea</i> (8), <i>sec/I</i> (3), <i>lukF/S-PV</i> (1), <i>tst</i> (13), egc (14), IEC- A(7), B(8), C(1), D(1), E(1), <i>icaA/C/D</i>	<i>sea</i> (6), <i>sec/I</i> (2), <i>tst</i> (10), egc (11), IEC- A(5), B(4), D(1), <i>icaA/C/D</i>

Genotype	n	Antimicrobial resistance ¹ (n) ²		Antimicrobial resistance genes (n) ²		Virulence genes (n) ²	
		2006/07	2011	2006/07	2011	2006/07	2011
ST34-MSSA	5	0	0	Ap, Cd	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>	N/A
CC45-MSSA	15	18	18	Ap (14), Cp (1), Er (1), Fd (1), Mp (1), Sp (2), Tb (1)	Ap (14), Cd(I) (8), Er (1), Fd (2), Gn (1), Kn (1), Tb (1)	<i>blaZ</i> (14), <i>sdrM</i> , <i>qacC</i> (1), <i>mupA</i> (1), <i>fusB</i> (2), <i>aadD</i> (1), <i>erm</i> (A) (2)	<i>blaZ</i> (14), <i>sdrM</i> , <i>tet</i> (K) (1), <i>aacA_aphD</i> (2), <i>fus</i> (B) (1), <i>erm</i> (A) (1)
CC59-MSSA	1	0	0	Ap	N/A	<i>blaZ</i> , <i>sdrM</i>	N/A
CC121-MSSA	2	1	1	Ap, Fd	Ap, Fd	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>farI</i>	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i> , <i>fus</i> (B)
CC188-MSSA	0	1	1	N/A	Ap, Cd(I)	N/A	<i>blaZ</i> , <i>sdrM</i>
CC361-MSSA	0	1	1	N/A	Ap, Cd(I), Cp, Er, Kn, Nm, Tp	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>msrA</i> , <i>mph</i> (C), <i>aphA3&sat</i> , <i>fosB</i>
ST34/42-MSSA	0	6	6	N/A	Ap, Cd	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>fosB</i>
ST72-MSSA	0	1	1	N/A	Ap, Er(I), Kn, Nm	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>aphA3&sat</i> , <i>fosB</i>
ST199-MSSA	0	1	1	N/A	Ap, Kn, Nm(I)	N/A	<i>blaZ</i> , <i>sdrM</i> , <i>aphA3</i> & <i>sat</i> , <i>fosB</i>

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Genotype	n	Antimicrobial resistance ¹ (n) ²		Antimicrobial resistance genes (n) ²		Virulence genes (n) ²	
		2006/07	2011	2006/07	2011	2006/07	2011
ST291/813-MSSA	0	3	N/A	Ap, Cd(I) (2)	N/A	<i>blaZ, sdrM</i>	<i>sea, etD & edinB, IEC-A, icaA/C/D</i>
ST1093-MSSA	0	1	N/A	Ap	N/A	<i>blaZ, sdrM</i>	<i>sea, tst, IEC-A, icaA/C/D</i>

¹ Susceptibility of isolates was determined to 23 antimicrobial agents including amikacin (Ak), ampicillin (Ap), cadmium acetate (Cd), chloramphenicol (Cl), ciprofloxacin (Cp), ethidium bromide, (Eb), erythromycin, (Er), fusidic acid, (Fd), gentamicin (Gn), kanamycin (Kn),, lincomycin (Ln), mercuric chloride (Mc), mupirocin (Mp), neomycin (Nm), phenyl mercuric acetate (Pma), rifampicin (Rf), sulphonamide (Su), spectinomycin (Sp), streptomycin (St), tobramycin (Tb), tetracycline (Te), trimethoprim (Tp), vancomycin.

² The number of isolates are indicated when not all isolates within a given genotype exhibited the same characteristic.

³ Immune evasion cluster (IEC) types were determined as described previously by Van Wamel *et al.*, (2008). A: *sea, sak, chp & scn*; B: *sak, chp & scn*; C: *chp & scn*; D: *sea, sak & scn*; E: *sak & scn*; F: *sep, sak, chp & scn*; G: *sep, sak & scn*.

⁴ N/A not applicable, no isolates of this genotype identified during the study period indicated.

Additional discrepancies were also detected between the phenotypic and genotypic results as determined by antimicrobial susceptibility testing and the DNA microarray profiling, respectively. This included (i) detection of the MLS_B resistance genes *erm(A)* and/or *erm(C)* among isolates exhibiting erythromycin resistance but susceptibility to lincomycin (MRSA: 2006/07, 54 isolates & 2011, 43 isolates ; MSSA: 2006/07, seven isolates & 2011, nine isolates), (ii) detection of trimethoprim resistance in the absence of *dfrS1* (other trimethoprim resistance genes are not detected using the DNA microarray) (MRSA: 2006/07, two isolates; MSSA: 2006/07, one isolate) and (iii) detection of *qacA* and/or *qacC* among isolates exhibiting susceptibility to the QAC ethidium bromide (MSSA: 2006/07, five isolates; 2011, two isolates) (Tables 5.3 and 5.4).

5.3.3 Virulence genes

The MRSA isolates exhibited similar rates of carriage of virulence genes across both study periods including *egc* (2006/07: 90.4% 75/83; 2011: 90.4%, 66/73), *IEC* (2006/07: 96.4%, 80/83; 2011: 100%, 73/73), *sec/l* (2006/07: 42%, 35/83; 2011: 37%, 27/73), *sea* (2006/07: 12%, 10/83; 2011: 9.6%, 7/73), *tst* (2006/07: 3.6%, 3/83; 2011: 1.4%, 1/73), *ACME-arc* (2006/07: 1.2%, 1/83; 2011: 2.7%, 2/73) and *sed/j/r* (2006/07: 3.6%, 3/83; 2011: 2.7%, 2/73) (Fig 5.3a). No virulence genes were uniquely identified in 2011 but two virulence gene clusters were unique to 2006/07 (*etD* & *edinB* and *ORF CM14*), but these were represented by only one isolate each (Fig. 5.3a).

The MRSA isolates harboured six of the seven previously identified IEC types (A-D, F and G) (Fig. 5.4) (vanWamel *et al.*, 2006) with IEC type B (*sak*, *chp* & *scn*) predominating during both study periods (2006/07: 85.5%, 71/83; 2011: 78.1%, 57/73). The majority of CC/ST22-MRSA-IV isolates during both study periods harboured IEC genes (2006/07: 95.8%, 68/71; 2011: 92.2%, 59/64) and of those that did, 100% carried IEC type B in 2006 and 98.3% in 2011 (Table 5.3). The remaining CC/ST22-MRSA-IV isolates harboured *sak* only and could not be assigned an IEC type. There were no significant differences in IEC gene carriage between both study periods for the MRSA.

The only statistically significant difference in the rate of carriage of virulence genes among the MRSA isolates between both study periods was for the biofilm-associated intercellular adhesion cluster gene *icaC*, which forms part of the *ica* gene cluster comprising *icaA*, C, D & B; *icaB*, C and D can be detected using the DNA

microarray (Fig. 5.3). In 2006/07, *icaC* was detected in 94% (67/71) of CC/ST22-MRSA-IV with the remaining 6% (4/71) yielding no signal or an ambiguous signal for *icaC* using the DNA microarray. However, in 2011, *icaC* was only detected in 50% (32/64) of CC/ST22-MRSA-IV isolates with the remaining 50% yielding no signal or an ambiguous signal for this gene (Fig. 5.3). This increase in MRSA isolates yielding negative or ambiguous signals for *icaC* was statistically significant ($p = 0$). In contrast, *icaC* was detected together with *icaB* and *icaD* in all other isolates except two CC22-MSSA isolates in 2011 which also yielded negative signals for *icaC* (Table 5.4).

Overall, more virulence genes were detected among the MSSA isolates compared to the MRSA isolates (Fig. 5.3). One virulence gene was unique to the MRSA isolates, the ACME-*arc* gene cluster, and four unique to MSSA including *lukF/S-PV*, *etA*, *etB* and *edinC* but, in each case all were detected in five isolates or less and the differences were not statistically significant (Fig. 5.3). ACME-*arc* was detected in ST22-MRSA-IV isolates only (Tables 5.3 and 5.4).

Of the virulence genes/gene clusters identified, 11/14 were common to MSSA from both study periods but for some of these genes the rates of carriage differed between the study periods (Fig. 5.3b). The IEC was the most common virulence gene cluster detected among the MSSA isolates during both study periods (2006/07: 95.2%, 80/84; 2011: 92.1%, 105/114) and the seven previously identified IEC types were detected among these isolates (Fig. 5.4). The IEC type E was unique to the MSSA isolates (2006/07: 6%, 5/84; 2011: 7%, 8/114) and was detected in genotypes CC1, CC5, CC8, CC10, CC30, CC121 and CC361 (Table 5.3). Similar to the MRSA IEC type B (*sak*, *chp* & *scn*) was the most common IEC identified among the MSSA although its prevalence decreased significantly among the MSSA between the two study periods (2006/07: 51.2%, 43/84; 2011: 36%, 41/114) ($p = 0.0348$). There was an increase in the carriage of IEC type G (*sep*, *sak*, *scn*) among MSSA between 2006/07 and 2011 (2006/07: 4.7%, 4/84; 2011: 1.5%, 12/114), although this was not statistically significant.

The second most common gene cluster detected among the MSSA was the enterotoxin gene cluster *egc*. The rate of carriage of *egc* decreased significantly from 72.6% (61/84) in 2006/07 to 55.3% (63/114) in 2011 ($p = 0.012$) (Fig. 5.3b). This was attributable to a decline in six *egc*-positive MSSA genotypes (CC20, CC25, CC30, ST34, CC45 and CC121) between the two study periods (Table 5.4). The prevalence of *sea*

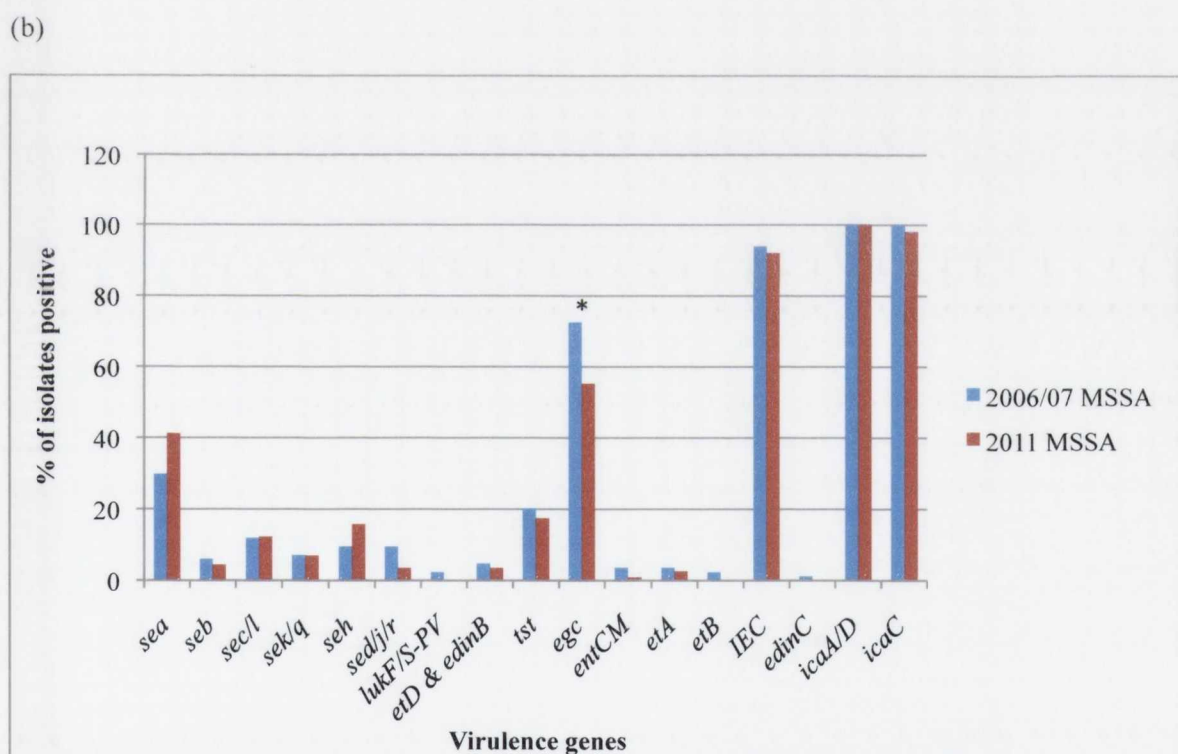
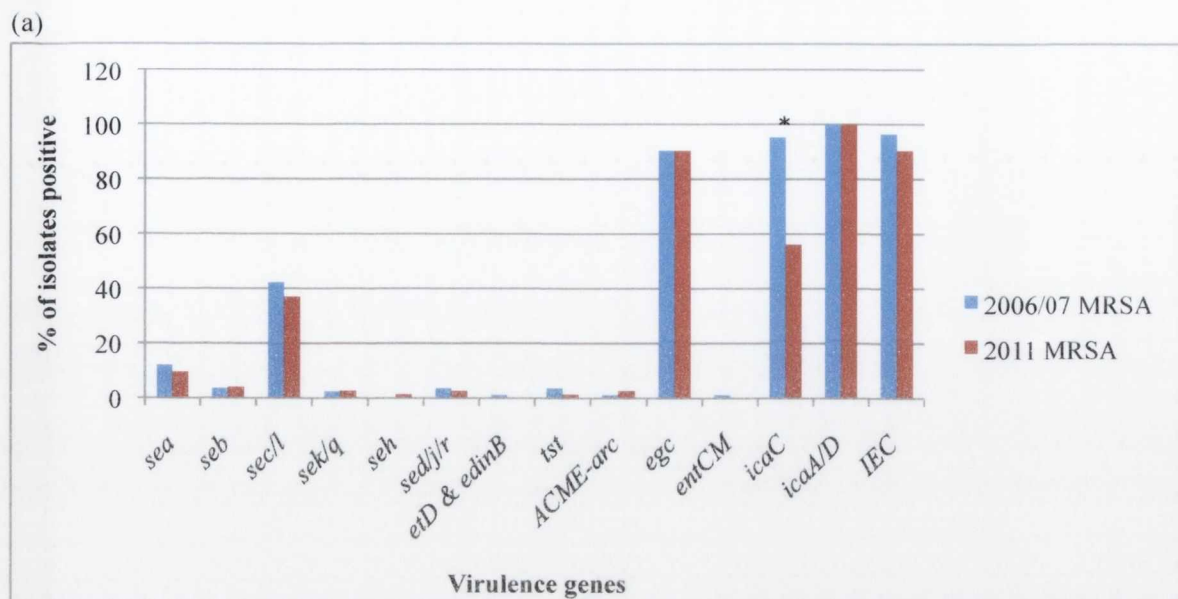


Figure 5.3 Virulence genes detected using the StaphyType DNA Microarray (Alere) among (a) MRSA and (b) MSSA isolates recovered from patients with bloodstream infections in Irish hospitals during two six-month periods in 2006/07 and 2011. Only genes which the isolates were positive for are shown. Table 2.4 shows a full list of virulence genes that can be detected by the DNA microarray and what they encode. Statistically significant differences are indicated with an asterisk (*).

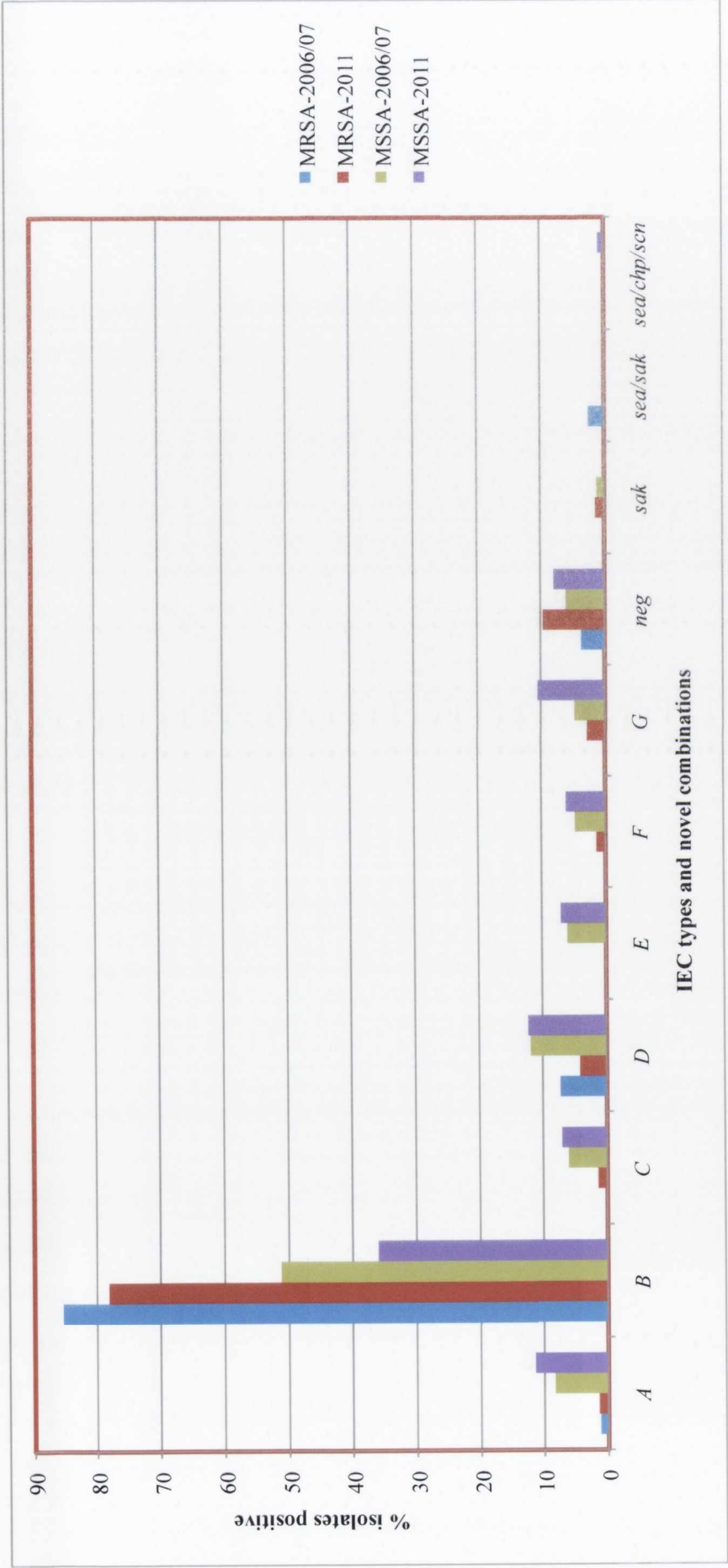


Fig. 5.4 Percentage of MRSA and MSSA isolates recovered from bloodstream infection in Irish hospitals during two study periods in 2006/07 and 2011 positive for each immune evasion cluster (IEC) type.

IEC types: A: *sea, sak, chp & scn*; B: *sak, chp & scn*; C: *chp & scn*; D: *sea, sak & scn*; E: *sak & scn*; F: *sep, sak, chp & scn*; G: *sep, sak & scn*. IEC types A-G were assigned as described previously by van Wamel *et al.*, (2006).

increased from 29.8% (25/84) in 2006/07 to 41.2% (47/114) in 2011 but this was not statistically significant. The increase in *sea* in 2011 was due to the increasing prevalence of the MSSA genotypes CC1 (three isolates in 2006/07 and 11 in 2011) and CC7 (two isolates in 2006/07 and nine in 2011) which harboured *sea* (Table 5.4). Other virulence genes detected among the MSSA isolates included *sec/l*, *sek/q*, *seh*, *sed/j/r*, *etA/B*, *etD* & *edinB* and *tst*, but there was no significant difference between the carriage of these virulence genes between isolates recovered during the two study periods (Fig. 5.3b). Three virulence genes were unique to the 2006/07 study period including *etB* (2.4%, 2/84), *edinC* (1.2% 1/84) and *lukF/S-PV* (2.4%, 2/84) and none were unique to 2011.

5.3.4 Patient Demographics

Details of patients from which MRSA and MSSA isolates were recovered from during both study periods are shown in Table 5.5. There was no significant difference between HO - MRSA or MSSA isolates between both study periods (MRSA: 53% and 46% 2006/07 and 2011 respectively; MSSA: 46% 2006/07 and 47.6% 2011). While there was no significant difference in the prevalence of either HO- or CO-MRSA, or HO-MSSA, during the two study periods, there was an increase in the rate of CO-MSSA in 2011 (52.3%) compared to 2006/07 (41%) but this was not statistically significant.

The 14 day mortality rate for patients was higher for those with MRSA infections (2006/07: 16.66%, 14/84; 2011: 13.7%, 10/73) compared to those with MSSA infections (2006/07: 13.2%, 7/84; 2011: 8.8%, 10/114) during both study periods but this was not statistically significant. There was no significant change in the 14-day mortality rate for those with MRSA or MSSA between 2006/07 and 2011.

Among MRSA and MSSA-positive patients there were more males than females in each group across both study periods. While 53.6% (45/84) of patients from whom MRSA isolates were obtained in the 2006/07 study period were male, this increased to 76.7% (56/73) of patients in 2011 and this was statistically significant ($p = 0.000252$). Similarly, among the MSSA patients, 53.6% (56/73) were male in 2006/07 and this increased in the 2011 study period to 64% (73/114) but this was not statistically significant.

The MSSA isolates were recovered from patients who were significantly younger than those from which the MRSA isolates were recovered from i.e. in 2006/07 75%

(63/84) of patients from whom MRSA was recovered were aged 60 years or older while only 52.4% (44/84) of MSSA isolates were recovered from patients aged 60 years or older in 2006/07. The average age of patients from whom MRSA was recovered decreased from 67.4 in 2006 to 66 in 2011. For MSSA there was also a decrease in the average age of patients from 51 in 2006/07 to 49.1 in 2011. However, neither decrease was found to be statistically significant.

Table 5.5 Patient demographics associated with MRSA and MSSA isolates recovered from patients with bloodstream infections in Irish hospitals during two six-month periods in 2006/07 and 2011

Patient demographics	No. of isolates			
	MRSA		MSSA	
	2006/07	2011	2006/07	2011
Hospital onset ¹	44	34	39	50
Community onset ¹	25	31	28	55
Unknown	14	8	17	7
14 day mortality²				
Living	51	42	65	94
Deceased	14	10	7	10
Unknown	19	21	12	10
Gender³				
Male	45	56	45	73
Female	39	17	39	41
Age (years)³				
0-9	2	2	11	25
10-19	1	0	5	3
20-29	1	1	5	2
30-39	3	4	4	10
40-49	4	5	6	9
50-59	10	11	9	10
60-69	17	11	16	20
70-79	23	18	15	12
80-89	21	18	13	19
90-99	2	3	0	4

¹Isolates were considered hospital-onset if symptoms developed more than 48 h after admission or community-onset if symptoms were present upon or within 48 h of admission.

²The fourteen day mortality refers to the number of patients deceased or surviving 14 days after recovery of the first isolate.

³Data on age and gender for each patient from which MRSA and MSSA were recovered were available for all isolates.

5.4 Discussion

This study has revealed several molecular epidemiological changes that may have contributed, at least in part, to the changing dynamics of MRSA and MSSA BSIs in Irish hospitals between 2006/07 and 2011. Despite a decline of almost 50% in MRSA BSIs between 2006/07 and 2011, ST22-MRSA-IV continues to predominate. When the ST22-MRSA-IV isolates recovered during both study periods were compared no statistically significant differences were identified in terms of *spa* types or antimicrobial resistance genes carried or in antimicrobial resistance phenotype, but a 44% increase was detected in the number of ST22-MRSA-IV isolates yielding negative or ambiguous signals for *icaC*, despite the detection of *icaA* and *icaD*. In contrast, several differences were identified between the MSSA isolates recovered during both study periods including an increase in the heterogeneity of the genetic backgrounds of the isolates, an increase in community-onset MSSA in younger adults, an increase in the rate of carriage of *sea* and a decrease in the rate of carriage of *egc*, *fosB* and IEC type B.

The *icaC* gene forms part of the intercellular adhesion cluster (ICA) which plays a role in biofilm formation among *S. aureus* (O’Gara J, 2007). However, a number of recent studies have revealed that while the ICA pathway is used for biofilm formation in MSSA, that alternative biofilm formation pathways are used by MRSA. Pozzi *et al.*, (2012) revealed that methicillin resistance and the presence of the *mec* gene affects *icaADBC*-mediated biofilm formation, with MRSA expressing alternative biofilm formation pathways such as those involving fibronectin binding proteins. McCarthy *et al.*, (2015) subsequently revealed that the *ica*-mediated biofilm pathway is completely redundant in MRSA with strains utilising biofilm formation pathways involving the release of extracellular DNA and cell surface expression of a number of sortase-anchored proteins and the major autolysin. Therefore, it is perhaps not surprising that ST22-MRSA-IV isolates may be acquiring mutations in, or losing components of, the redundant *ica* pathway. The lack of detection of *icaC* in the ST22-MRSA-IV isolates in the present study may be due to mutations in the DNA microarray primer or probe binding sites. This may be indicative of more genome-wide SNVs within the more recent ST22-MRSA-IV isolates. In this regard further studies are required to determine the reason for the lack of the *icaC* signal in the more recent ST22-MRSA-IV isolates. Additional studies were undertaken in order to investigate this further and are described in Chapter 6. This included whole-genome sequencing studies to compare the numbers and positions of

SNVs within ST22-MRSA-IV isolates from both study periods and an investigation of a possible fitness cost associated with any SNVs identified that may provide evidence of clonal decline. The absence of a signal for *icaC* in a single CC22-MSSA isolate in the present study may reflect that this isolate evolved from a MRSA due to the loss of *SCCmec* and therefore the ICA pathway was redundant as all other CC22-MSSA isolates were positive for *icaC*.

Clonal decline has been implicated previously in the changing epidemiology of MRSA among BSIs (Albrecht *et al.*, 2011; Rao *et al.*, 2011). For example, Ellington *et al.* (2010) identified different epidemic curves of the two main MRSA clones in the UK, ST22-MRSA-IV (EMRSA-15) and ST36-MRSA-II (EMRSA-16), and the corresponding peaks and declines in BSI rates associated with both clones. In that study EMRSA-15 and EMRSA-16 together consistently accounted for approximately 95% of MRSA causing BSIs in the UK between 2001 and 2007, but the proportions of EMRSA-16 declined significantly, whilst the proportion of EMRSA-15 rose commensurately. The authors speculated that antimicrobial prescribing policies, improved infection control or bacteriophage epidemics may play a role in the decline of once predominant clones.

An increasingly polyclonal MSSA population was identified among BSIs in 2011. Since the emergence of MRSA strains occurs via the acquisition of *SCCmec* elements by MSSA, MRSA strains are much younger in evolutionary terms than MSSA and tend to be less diverse than MSSA. The broader distribution of the many MSSA clones identified here and the lack of a single dominant clone across the study periods may be due to the different population dynamics of the younger MSSA patients or the fact that the MSSA clones have had a longer time to diversify. Even within the MSSA clones more genotypic diversity was identified than among MRSA clones. An example of this can be seen among the IEC types carried by the clones in this study. MRSA clones typically harbour just one IEC type per clone with a few exceptions mainly due to novel variants. The majority of the MSSA clones in the present study however each carried more than one IEC type, up to five in some cases, highlighting the diversity even within MSSA isolates belonging to the same genotypes. This increasing diversity among the MSSA isolates could lead to a fitness advantage over the MRSA isolates with MSSA isolates better able to compete within the changing environments and population dynamics of BSI patients compared to the relatively limited homogenous clones of MRSA which could be undergoing clonal decline.

The increasing prevalence of CO-MSSA BSIs during the study period suggests that MSSA carriage in the community is playing an important role in the epidemiology of *S. aureus* BSIs. Grundmann *et al.*, (2014) highlighted the now European-wide trend of significant increases in CO-*S. aureus* BSIs. That study revealed significant increases in both CO-MRSA and CO-MSSA unlike the present study where only an increase in CO-MSSA BSIs were identified.

The 24% decrease in the rate of carriage of the superantigen enterotoxin gene cluster *egc* identified among the MSSA isolates between 2006/07 (72.6%, 61/84) and 2011 (55.3%, 63/114) was predominantly associated with the decline of six specific MSSA clones. However, unlike other staphylococcal superantigen toxins such as *sea* or *tst*, the presence of *egc* is negatively correlated with the severity of infections, with higher rates of carriage of *egc* reported among *S. aureus* isolates from nasal carriage than invasive infections (Holtfreter *et al.*, 2004; Ferry *et al.*, 2005). Therefore, it seems unlikely that the decrease in the prevalence of *egc* among the MSSA isolates in 2011 would contribute negatively to the virulence of MSSA which correlates with the increasing prevalence of MSSA BSIs. There were also significant declines in the prevalence of the fosfomycin resistance gene *fosB* and the IEC type B genes among the MSSA. In both cases the decline was associated with a decline in the specific clones harbouring these genes within the study period, which could be reflective of the increased heterogeneity of MSSA clones identified.

Although not statistically significant an increase in the prevalence of another superantigen enterotoxin gene, *sea*, was identified among the MSSA isolates in 2011. The *sea* gene encodes a potent superantigen linked to severe systemic manifestations of *S. aureus* infection such as sepsis and induces strong pro-inflammatory responses (Dauwadder *et al.*, 2006; Schelin *et al.*, 2011; Cao *et al.*, 2012). Further research is required using additional and more recent MSSA isolates to determine if this increase in the prevalence of *sea* is an ongoing trend among MSSA causing BSIs in Ireland. Further work could also investigate levels of expression of *S. aureus* toxins among *S. aureus* BSI isolates during different study periods to determine if increases in expression of *sea*, or other toxins, are associated with an increasing prevalence of MSSA BSIs.

The decline in MRSA and increase in MSSA among *S. aureus* BSIs in Ireland is reflective of what has also been identified across Europe (deKraker *et al.*, 2012;

Grundmann *et al.*, 2014). Little difference in the dominant MSSA and MRSA clones causing *S. aureus* BSIs were identified across Europe between 2006 and 2011 (Grundmann *et al.*, 2014). Across Europe ST22-MRSA-IV was the most common clone causing MRSA BSIs in both 2006/07 and 2011 although a significant increase was detected in the number of *spa* types associated with this clone in 2011 compared to 2006 (Grundmann *et al.*, 2014). This is in contrast to what was found in the present study where no significant increases in ST22-MRSA-IV *spa* types were detected. Among MSSAs causing BSIs in Europe the same top ten *spa* types were identified during both study periods with changes only in rank order (Grundmann *et al.*, 2014). This is similar to what was identified in the present study where CC30, CC45 and CC5 predominated during both study periods but to differing degrees. Grundmann *et al.* (2014) identified a similar patient profile and demographics of *S. aureus* BSIs throughout Europe as was identified here, with BSIs associated with a predominantly older male patient.

Improvements in MRSA-specific hospital infection control programmes or more targetted antimicrobial prescription practises could also play a role in the decline of MRSA among BSIs. In this study however isolates were recovered from patients from a wide geographic region in Ireland so it is unlikely that any single hospital-specific policy could influence the results to such an extent and levels of MSSA are not declining to the extent of MRSA which would be expected if an overall national infection control policy was causing the decline.

Similar to other reports this study reinforced the concept that trends towards ageing and community acquisition have affected the demographics of patients suffering from *S. aureus* BSIs (Gasch *et al.*, 2011; Gasch *et al.*, 2012). This study was unable to identify if certain clones were associated with increased mortality among the MRSA BSIs as numbers of clones other than ST22-MRSA-IV were too low. Similarly among the MSSA, although CC30-MSSA was associated with greater mortality than any other clone, numbers of isolates were too low to identify a definitive association between specific clones and mortality rates that has been seen in other studies (Holmes *et al.*, 2011; Gasch *et al.*, 2012).

The MSSA isolates harboured more virulence genes than the MRSA isolates as has been shown in other studies (Rodríguez *et al.*, 2014; Jiménez *et al.*, 2011). Since the acquisition of additional antimicrobial resistance determinants, and in some cases

mutations encoding resistance, are associated with reduced fitness (Rudkin *et al.*, 2012), MRSA isolates may compensate by harbouring less virulence-associated genes. Despite this, in the present study patients with MRSA had a higher mortality rate than those with MSSA. Patients with MRSA infections were also significantly older than those with MSSA BSIs suggesting that this increased virulence is responsible for the propensity of these strains to cause infection in otherwise healthy young patients. It has been suggested that the presence of numerous virulence determinants predicts, to a certain extent, the ability of colonising isolates to cause infections (McClure *et al.*, 2014) and this, coupled with the increases in CO-MSSA identified in this study, highlight the pathogenic potential of MSSA isolates in the community. MRSA isolates were associated with more infections in the older age group and with a higher mortality rate than MSSA and this may be due to other comorbidities associated with this cohort as opposed to increased virulence of these strains. The increased mortality rate compared to MSSA may also be due to resistance and the failure of initial therapy.

Ten CCs were common to both the MRSA and MSSA isolates in the present study. Within these, *spa* typing revealed an increase in the possible local emergence of MRSA from MSSA, or *vice versa*, due to an increase in the number *spa* types (n=5) common to both MRSA and MSSA between 2006/07 and 2011. Two of these *spa* types belonged to CC22-MRSA-IV, this may reveal further evidence of the clonal decline of ST22-MRSA-IV with isolates possibly losing *SCCmec* due to fitness costs associated with its carriage. Certain clones showed different characteristics depending on whether they were MRSA or MSSA, highlighting the diversification of individual clones within a given niche. In this study CC8-MSSA harboured more resistance genes and were resistant to more antimicrobial agents than the CC8-MRSA isolates, indicating that the CC8-MSSA may act as a reservoir for future dominant CC8-MRSA clones. Alternatively the CC8-MSSA isolates may have derived from the dominant CC8-MRSA clone as has been previously identified for multiresistant CC8-MSSA from Ireland (Shore *et al.*, 2008). The CC22-MSSA in this study in contrast were much less resistant than their CC22-MRSA counterparts, possibly mediated in part by the fact that CC22-MRSA in Ireland is predominantly a nosocomial clone in an antibiotic rich environment.

The study does have some limitations. Isolates were only collected during two specific study periods and a further collection of isolates would illustrate if the observed trends were continuing to the present day. The study collected no information on

precursors to infection in patients thus making it difficult to identify if disease outcome or patient profiles are influenced by underlying health issues. However, despite this, the present study did benefit from significant geographic breadth within Ireland. Another limitation of the study was that discrepancies were detected between the phenotypic antimicrobial resistance profiles and antimicrobial resistance genes detected in isolates using the DNA microarray. However, it is likely that this was due to a large extent to the presence of alternative resistance genes in these isolates which were not detectable using the array but which result in the phenotypic resistance patterns observed. Another reason could be lack of CLSI or EUCAST breakpoints for some agents used in the AR panel which was developed in house by the NMRSARL and may not be as robust as other published criteria.

In conclusion, this study has revealed that MRSA are still responsible for a significant number of BSIs with a high mortality rate and a typically older patient profile. The ST22-MRSA-IV clone is still the predominant cause of MRSA BSIs. The reason for the lack of detection of *icaC* among 50% of the CC22-MRSA-IV isolates in 2011 requires further investigation to determine if this has potentially contributed to the decline of CC22-MRSA-IV in Irish hospitals or if it is indicative of more genome-wide SNVs. MSSA are the cause of an increasing burden of BSIs. A shift towards more CO-MSSA infections in younger adults suggests that this population could be an important reservoir for MSSA. Targeting specific strains, such as MRSA clones with specific antibiotic therapy or the hospital environment with infection control measures may not significantly alter the numbers of MSSA BSIs and community-based interventions should be investigated. Temporal changes in the dominant MSSA lineages have occurred and there is an increasing diversity of MSSA lineages and a potential increase in the local emergence of MRSA from MSSA or *vice versa*. There is evidence of enhanced virulence potential among the MSSAs from the increasing prevalence of *sea* and the significant decline in *egc*. Future interventions should take into consideration the ability of MSSA BSIs to cause serious infections in young adults and children in the community setting.

Chapter 6

ST22-MRSA-IV in Ireland

6.1 Introduction

The reasons for the success and dominance of MRSA as a pandemic pathogen are multifaceted and include: 1) the initial acquisition of *mecA* by MSSA in the 1960's and the subsequent acquisition of additional resistance determinants resulting in multidrug resistance, 2) the emergence and subsequent global spread of pandemic nosocomial MRSA clones and 3) the emergence, divergence and success of MRSA in the community and animals (Klein *et al.*, 2007; DeLeo & Chambers, 2009; Grundmann *et al.*, 2010; Ito *et al.*, 2012; Otto, 2013, van Cleef *et al.*, 2015). *Staphylococcus aureus* is capable of existing in a wide range of environments. From the nares of humans to contact surfaces, companion animals, livestock and food sources, *S. aureus* has adapted and responded to each new biological niche. The ongoing success of this bacterium requires constant adaptation to new stresses and environments, a complicated process involving, amongst other things, the emergence of new clones, the replacement of existing dominant clones and the rapid dissemination of these successful clones (deLeo & Chambers, 2009; Grundmann *et al.*, 2010).

The cyclical emergence, expansion and decline of epidemic clones is a key factor in the isolation rates of clonal bacteria (deLeo & Chambers, 2009; Albrecht *et al.*, 2011). Irish molecular epidemiological typing data shows the sustained peaks and declines of MRSA clones isolated from patients in hospitals predominating at different time periods in Ireland (Shore *et al.*, 2005; Shore *et al.*, 2010). Serial displacement, clonal rise and fall of *S. aureus* clones have also been described in the UK and Europe (Ellington *et al.*, 2011; Espadinha *et al.*, 2013). The causative factors behind clonal expansion and decline are multifaceted and have been reported to include bacterial genetics, strain competition, host immune response, infection prevention and control measures and screening (Section 1.4).

Clonal replacement of the predominant MRSA lineages circulating in Irish hospitals has been identified many times over the last forty years (Shore *et al.*, 2010; Shore *et al.*, 2005). ST250-MRSA-I/I-*pls* predominated in the 1970s until ST239-MRSA-III/III-p1258/Tn554 replaced it in the mid to late 1980s and continued to be the dominant clone into the 1990s until ST8-MRSA-IIA-IIIE overtook it. By the late 1990s there were three clones predominating, ST8-MRSA-IIA-IIIE, ST36-MRSA-II and ST22-MRSA-IV, the latter of which has become the most successful clone in Irish hospitals

accounting for up to 80% of MRSA BSIs yearly from 2003-2014 (NMRSARL, 2014). The ST22-MRSA-IV clone has, since its first isolation in the UK in 1991 (Richardson & Reith 1993), become associated with a global epidemic causing infections across the continents (Holden *et al.*, 2013). A recent European-wide study of *S. aureus* isolates causing BSIs found ST22-MRSA-IV to be the most frequently identified clone (Grundmann *et al.*, 2010). The success of the ST22-MRSA-IV clone has been well documented and its ability to replace existing predominant clones has been identified elsewhere including replacement of ST288-MRSA-I in a large hospital in Milan, Italy (Baldan *et al.*, 2015), the replacement of ST239-MRSA-III in Portuguese hospitals (Aires-de-Souza *et al.*, 2010) and replacement of CC5-MRSA-I and CC45-MRSA-IV in a German University hospital (Albrecht *et al.*, 2010) amongst others (Ellington *et al.*, 2010; Aires-de-Souza *et al.*, 2008; Witte *et al.*, 2008; Hsu *et al.*, 2005).

Successful *S. aureus* lineages can exchange not only antimicrobial-agent resistance genes but also virulence-associated genes leading to new hypervirulent multiresistant strains capable of causing serious infections in healthy individuals (Bernal *et al.*, 1999; vanWamel *et al.*, 2006; Prabhakara *et al.*, 2012). The success of ST22-MRSA-IV has been attributed predominantly to a series of gene acquisitions and mutations associated with antibiotic resistance which enabled it to thrive in a hospital setting (Holden *et al.*, 2013). Holden *et al.*, (2013) identified two non-synonymous SNVs in ST22-MRSA-IV isolates associated with fluoroquinolone resistance which support the successful emergence and expansion of this clone within the hospital setting. Fluoroquinolones were introduced into routine clinical practice in 1987 in the UK and ST22-MRSA-IV emerged in 1991 with its rapid spread coinciding with the increased usage of fluoroquinolone antibiotics throughout hospitals and the community in the UK (Holden *et al.*, 2013; Livermore *et al.*, 2002). However, fluoroquinolone resistance alone has not lead to the pandemic success of ST22-MRSA-IV and mutations in virulence genes such as the extracellular matrix-binding protein *ebh* and fibronectin binding proteins *fnbA/B* have also been implicated (Holden *et al.*, 2010; Clarke *et al.*, 2002). Additionally, the presence of ACME in some ST22-MRSA-IV isolates could potentially lead to enhanced virulence in this strain by improving its ability to grow and survive on the skin but its prevalence among ST22-MRSA-IV isolates appears to be relatively low (Shore *et al.*, 2011).

Although ST22-MRSA-IV has accounted for 70-80% of *S. aureus* BSIs in Ireland over the last decade the numbers of MRSA causing BSIs declined from 41.9% in 2006 to 27.1% in 2009, 24% in 2011 and 20.3% in 2013 (Chapter 5, Table 5.1). An investigation into the genotypes, virulence genes and antimicrobial resistance gene content of *S. aureus* isolates causing BSIs in Ireland in 2006/07 and 2011 identified ST22-MRSA-IV as the predominant clone in both study periods accounting for 85.5% of MRSA BSIs in 2006/07 and 87.7% in 2011 (Chapter 5). Figure 6.1 highlights the virulence and antimicrobial resistance gene content of ST22-MRSA-IV isolates recovered from BSIs in Ireland in 2006/07 ($n = 83$) and 2011 ($n = 73$) (Chapter 5). The only significant difference between isolates recovered during the two study periods as determined by DNA microarray profiling was in the detection of *icaC*, an intercellular adhesion cluster gene which plays a role in biofilm formation in *S. aureus*. The *icaC* gene was detected in almost 50% less isolates in 2011 than in 2006/07, while the remaining two genes of the cluster detected by the microarray, *icaA/D*, were detected in all ST22-MRSA-IV isolates in each study period. The lack of detection of *icaC* could be due to mutations in, or loss of, the gene by more recent ST22-MRSA-IV isolates. Due to its importance in biofilm formation, mutations in *icaC* could be indicative of further genome-wide mutations in ST22-MRSA-IV and a detailed analysis of these SNVs could provide possible evidence of clonal decline and a causative factor for the reduction in the number of ST22-MRSA-IV BSIs detected in recent years in Ireland.

The reasons for the decline of ST22-MRSA-IV among *S. aureus* BSIs in Ireland are still unclear. The reduction in the prevalence of *icaC* among ST22-MRSA-IV isolates in 2011 as detected by microarray profiling requires further investigation to determine if this has contributed to the decline or if it is indicative of further genome wide SNVs and possibly clonal decline. Therefore, the purpose of this part of the present study was to undertake a comparative analysis of ST22-MRSA-IV isolates recovered in 2006, 2009 and 2011 in order to identify factors that may have contributed to the decline in the incidence of ST22-MRSA-IV among BSIs in Ireland in recent years. A three-way approach was undertaken to analyse the ongoing fitness of the Irish ST22-MRSA-IV clone by comparing BSI isolates recovered during 2006, 2009 and 2011 using (i) DNA microarray profiling to determine if the increase in the lack of detection of *icaC* identified in more recent ST22-MRSA-IV isolates could be detected in additional isolates from 2006 and 2009/10, (ii) growth curves to identify if there was a reduction in growth rate

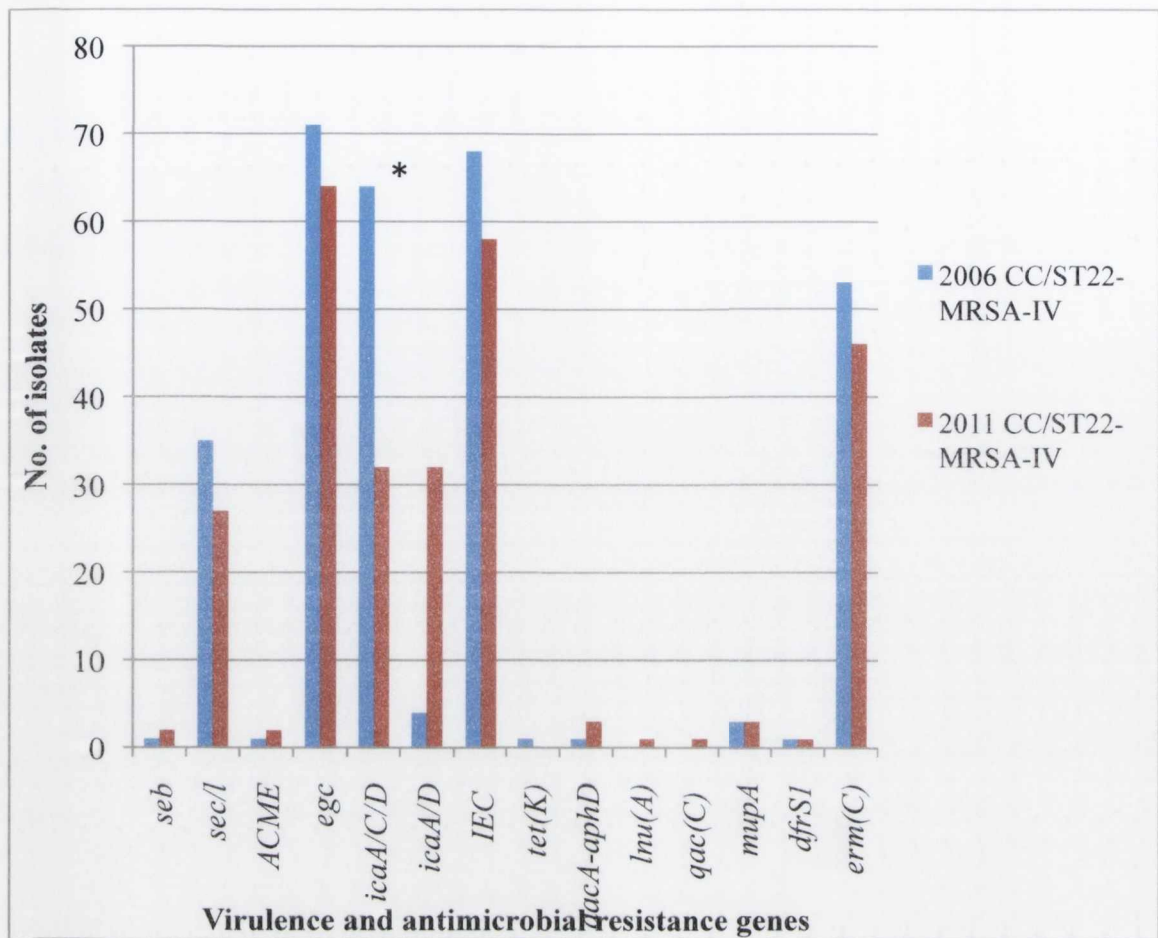


Figure 6.1 The virulence and antimicrobial resistance gene content of ST22-MRSA-IV isolates recovered from patients with BSIs in Ireland in 2006/07 ($n = 83$) and 2011 ($n = 73$), respectively, determined by DNA microarray profiling. *icaA/C/D* includes all isolates which had all three of the *ica* locus genes. *icaA/D* includes isolates which harboured *icaA* and *icaD* but lacked *icaC*.

Differences marked with an asterisk (*) are statistically significant.

that may indicate clonal decline in more recent isolates, and (iii) whole genome sequencing (WGS) to investigate the genetic relatedness of isolates from both study periods to identify any unique SNVs (or missense mutations) that could be linked to the decline in numbers of MRSA recovered from BSIs in Ireland and possible clonal decline.

6.2 Materials and Methods

6.2.1 Bacterial isolates

A total of 77 ST22-MRSA-IV isolates were investigated, all of which were recovered from patients with BSIs in Irish hospitals (Table 6.1). Forty-eight isolates underwent DNA microarray profiling, 24 each from 2006 and 2009/10. These isolates were selected to represent as diverse a range as possible of AR06 subtypes identified among ST22-MRSA-IV isolates during these time periods (Table 6.1).

Growth curves were performed on 34 ST22-MRSA-IV isolates, 17 from 2006/07, six from 2009 and 11 from 2011 (Table 6.1). These isolates were assigned to three groups (A-C, Table 6.1). Group A consisted of six isolates from 2006/07 and six from 2011 which were recovered from patients with BSIs in Irish hospitals as part of the European *spa* typing study. This group was selected such that isolates from both years were as similar to each other as possible in terms of *spa* type, antimicrobial resistance profiles and virulence and antimicrobial resistance gene content as determined in Chapter 5 (Table 6.1). Group B consisted of five isolates from 2006/07 and five from 2011 again collected as part of the European *spa* typing study. Here the isolates were selected to represent a diverse range of *spa* types, antimicrobial resistance phenotypes and virulence and antimicrobial resistance genes in each year as determined in Chapter 5 (Table 6.1). Group C consisted of six isolates each from 2006 and 2009/10. These isolates were also from BSIs and represented closely related AR06 isolates from both study periods (Table 6.1). Statistical analysis of growth curve data was performed using PRISM (GraphPad, 6.0).

Whole-genome sequencing and SNV analysis was undertaken on seven ST22-MRSA-IV isolates which had also undergone growth curve analysis (Table 6.1). The four fastest growing isolates from 2006/07 and the three slowest growing isolates from 2011 were selected for analysis (Table 6.1). This was undertaken to compare the SNV differences between these isolates and to compare their *icaC* gene content. The four isolates from 2006/07 were *icaC*-positive as detected by DNA microarray profiling and of the three from 2011, one was positive, one negative and one yielded ambiguous signals for *icaC* using the microarray.

Table 6.1 Details of ST22-MRSA-IV isolates investigated

Purpose of investigation	Isolates (n)	Year of isolation (n)	spa types (% indicated when not 100%)	AR type ¹ (n)	Antimicrobial resistance (% indicated when not 100%)	Virulence and resistance genes (% indicated when not 100%)	Reference chapter
DNA microarray profiling to compare the prevalence of virulence and antimicrobial resistance genes and to identify if lack of detection of <i>icaC</i> also seen in these isolates	EARS-Net (48)	2006 (24)	NA	06.1	Ap, Cd (62.5), Cp (95.8), Er (62.5), Fd (45.8)	NA	This chapter
				06.1 CpR FdR			
				06.3 CpR (3)			
				06.3 CpR FdR (2)			
				06.5 CpR (5)			
				06.5 CpR FdR (5)			
				06.6 CpR (2)			
				06.6 CpR FdR (3)			
Growth curves to compare the growth rate of isolates	Group A (12)	2006/07 (6)	t032	N/A	Ap, Cd (50), Cp, Er, Fd (16.7)	<i>blaZ</i> , <i>erm</i> (C), <i>sec/I</i> (50), <i>egc</i> , <i>IEC</i> , <i>icaC</i> , <i>icaA/D</i>	Chapter 5
				06.1			
				06.1 CpR (2)			
				06.1 CpR FdR			
				06.3 CpR (3)			
				06.3 CpR FdR (2)			
				06.5 CpR (5)			
				06.5 CpR FdR (5)			
				06.6 CpR (2)			
				06.6 CpR FdR (3)			
	Group B (10)	2006/07 (5)	t032 (60), t885 (20), t1865 (20)	N/A	Ap, Cd (40), Cp, Er (60)	<i>blaZ</i> , <i>erm</i> (C) (40), <i>sec/I</i> (60), <i>egc</i> , <i>IEC</i> , <i>icaC</i> , <i>icaA/D</i>	Chapter 5
				06.1			
				06.1 CpR (2)			
				06.1 CpR FdR			
				06.3 CpR (3)			
				06.3 CpR FdR (2)			
				06.5 CpR (5)			
				06.5 CpR FdR (5)			
				06.6 CpR (2)			
				06.6 CpR FdR (3)			

Purpose of investigation	Isolates (n)	Year of isolation (n)	<i>spa</i> types (% indicated when not 100%)	AR type ¹ (n)	Antimicrobial resistance (% indicated when not 100%)	Virulence and resistance genes (% indicated when not 100%)	Reference chapter
		2011 (5)	t032 (80), t2945 (20)	N/A	Ap, Cd (80), Cp, Er (80), Fd (60), Gn (20), Kn (20), Ln (20), Mp (40), Rf (20), Tb (20)	<i>blaZ</i> , <i>erm</i> (C) (60), <i>sec/I</i> (60), <i>lnu</i> (A) (20), <i>aacA_aphD</i> (40), <i>mupA</i> (40), <i>egc</i> , <i>IEC</i> , <i>icaC</i> (40), <i>icaA/D</i>	
	Group C (12)	2006 (6)	NA	06.1 CpR; 06.3 CpR; 06.5 CpR; 06.6 CpR (2); 06.6 CpR FdR	Ap, Cd (33.3), Cp, Er (66.7), Fd (16.7)	NA	This chapter
		2009/10 (6)	NA	06.1 CpR; 06.3 CpR; 06.5 CpR (2); 06.5 CpR FdR 06.6 CpR FdR	Ap, Cd (66.7), Cp, Er (66.7), Fd (33.3)	NA	

Whole-genome sequencing to compare SNV differences between isolates	BSI (7)	2006/07 (4)	t032	NA	Ap, Cp, Cd (75), Er, Fd (25)	<i>erm</i> (C), <i>egc</i> , <i>sec/I</i> (50), <i>IEC</i> <i>icaC</i> , <i>icaA/D</i>	Chapter 5
		2011 (3)	t032	NA	Ap, Cp, Cd (33.3), Er, Fd (66.7)	<i>erm</i> (C), <i>egc</i> , <i>sec/I</i> (66.7), <i>IEC</i> <i>icaC</i> (33.3), <i>icaA/D</i>	Chapter 5

¹Antibiogram-resistogram (AR) typing was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI 2006; Rossney *et al.*, 2007). All AR typing was carried out in the NMRSARL, Dublin, Ireland. MRSA isolates were assigned to an AR type or AR type variant based on the antimicrobial susceptibility pattern a panel of 23 antimicrobial agents and 46 AR type numbers have been assigned to date (AR01-AR46). AR subtypes were assigned based on slight variations to an existing pattern, e.g. if an isolate lost a plasmid encoded resistance gene, this new phenotype would be assigned an AR subtype by the addition of a third numerical digit to the AR number e.g. AR14.1. Variations of the same subtype i.e. AR06.1 AR06.1 CpR occur where there are one or two differences in the AR pattern of the same type. CpR, ciprofloxacin resistant, FdR, fucidic acid resistant.

²Susceptibility of isolates was determined to 23 antimicrobial agents including amikacin, ampicillin (Ap), cadmium acetate (Cd), chloramphenicol, ciprofloxacin (Cp), ethidium bromide, erythromycin, (Er), fusidic acid, (Fd), gentamicin (Gn), kanamycin (Kn), lincomycin (Ln), mercuric chloride, mupirocin (Mp), neomycin, phenyl mercuric acetate, rifampicin (Rf), sulphonamide, spectinomycin, streptomycin, tobramycin (Tb), tetracycline, trimethoprim and vancomycin.

³N/A not applicable, no results for this section available or data determined as part of the present study and results are shown later.

6.2.2 DNA microarray profiling

DNA microarray analysis of the 48 ST22-MRSA-IV isolates was performed as described in Chapter 2, section 2.4.4.

6.2.3 Comparison of the growth rate of isolates

Isolates were activated by removing a single bead from the cryostorage vial using a sterile inoculating loop and inoculating onto a BHI agar plate. Plates were incubated overnight at 37°C in a static incubator. Two to three colonies were selected from this overnight culture and were used to inoculate 20 ml of BHI broth in a 50 ml tube and grown overnight in a 37°C incubator with shaking at 200 rpm. This overnight bacterial suspension was then diluted using fresh BHI broth to form a stock solution with an optical density (OD) at 600_{nm} of 0.01 (determined using the Evolution 60S Spectrophotometer, Thermo Scientific). A 200 ml volume of this stock solution was inoculated into 20 ml of fresh BHI broth in a 50 ml tube. The broths were incubated at 37°C in a shaking incubator at 200 rpm for the duration of the experiment. One ml aliquots of each isolate were removed hourly for the first 8 h, including at time zero i.e. before it was incubated, and the OD₆₀₀ of each isolate was measured. A final OD measurement was taken after each isolate had been incubated for 24 h. Growth curves were performed, in triplicate on separate occasions with each isolate group's growth curves always performed together. Data was analysed using Microsoft Excel (2010) and Prism (GraphPad 6.0).

6.2.4 Whole-genome sequencing

Genomic DNA for WGS was extracted as described in Chapter 2 (section 2.4.1) using the Qiagen DNeasy kit (Crawley, UK). Nextera XT library preparation reagents (Illumina, Eindhoven, The Netherlands) were used to generate multiplexed paired-end sequencing libraries of each genomic DNA sample. This included enzymatic fragmentation of each genome with subsequent addition of indexed adapter sequences at either end of the resultant DNA fragments in a five step process (Fig. 6.2), each of which are described below in detail (sections 6.2.4.1-6.2.4.6). Resultant pooled libraries were sequenced and data was analysed as described in section 6.2.4.6.

6.2.4.1. Tagmentation

Genomic DNA was diluted to a concentration of 0.2 ng/ μ l using the Qubit 2.0 flourometer (Thermo Fischer Scientific). A 10 μ l volume of Tagment DNA Buffer (Illumina Nextera Kit) were added to each required well of a 96-well plate (Greiner bio-one, Austria). A 5 μ l volume of extracted genomic DNA at a concentration of 0.2 ng/ μ l was added to each well followed by 5 μ l of Amplicon Tagment Mix (Illumina Nextera Kit). Samples were pipetted up and down five times to ensure a homogenous mix. The plate was then sealed and centrifuged at 280 x g at 20°C for one min. The plate was then transferred to a thermocycler and held at 55°C for five min before being cooled to 10°C. A five μ l aliquot of Neutralisation Buffer (Illumina Nextera Kit) was immediately added to each sample well and pipetted up and down five times. The plate was again sealed and centrifuged for one minute at 280 x g at 20°C followed by incubation at room temperature for five min and immediate progression to the PCR amplification step.

6.2.4.2 PCR amplification

To avoid cross reactivity between samples and to label each sample with a genetic marker, Index1 and Index2 primers as supplied with the kit, were selected using Illumina Experiment Manager according to the manufacturer's guidelines (Illumina), one unique primer from each index per sample (Fig. 6.2). A 15 μ l aliquot of Nextera PCR Master Mix was added to each sample well. Then 5 μ l of index 2 primers were added to each well followed by 5 μ l of index 1 primers. Individual samples were mixed by pipetting up and down five times and then centrifuged for one min at 280 x g at 20°C. Samples were then transferred to a thermocycler for the following program: 72°C for 3 min; 95°C for 30 s; 12 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 30 s, and one final 72°C for 5 min step followed by a 10°C hold step.

6.2.4.3 PCR clean up

A 25 μ l aliquot of AMPure XP beads (Labplan, Naas, Kildare, Ireland) was added to each sample well and mixed by pipetting up and down 10 times. Samples were then incubated at room temperature for five min. The 96 well plate was then placed on a magnetic stand (Life technologies) until the beads, now hybridised to the amplified genome fragments, formed into a pellet and the supernatant was carefully removed and discarded. The beads were then washed twice with 80% (v/v) ethanol while still placed on the magnetic stand.

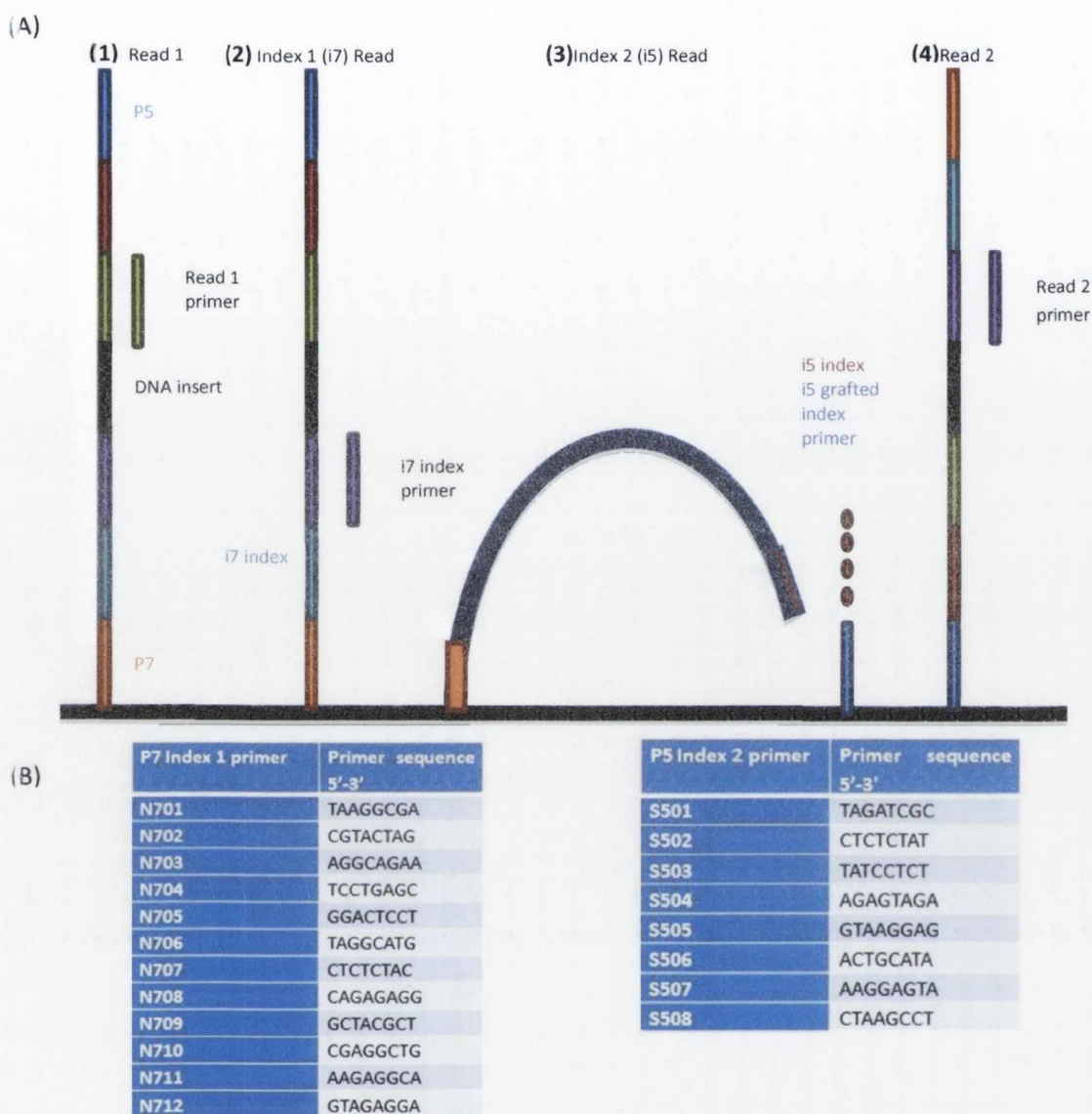


Figure 6.2 Stepwise description of the workflow involved in a paired-end dual-indexed MiSeq whole-genome sequencing run as adapted from Illumina (A) part (1): The library starts as single-stranded DNA fragments with P5 and P7 ends. The P5 and P7 regions anneal to their complementary oligos bound to the flowcell. These act as primers and a complementary strand to the library fragment is formed. (A) part (2): The original DNA strands are removed leaving behind the copies which are still bound to the flowcell in different orientations (only one strand is shown in diagram). The sequencing primer from Index 1 (i7) anneals to the template strand. The prepared index read then undergoes eight cycles of sequencing. (A) part (3): The i7 read product is removed, the P5 region is cleaved leaving only those fragments which are attached by the P7 region. To attach the i5 index to the i5 primer grafted to the flow cell seven chemistry only cycles take place followed by the eight cycles of sequencing. The index read product is then removed and the complementary strand is regenerated using the original template which is then removed allowing hybridisation of the Read 2 primer. (A) part (4): Read 2 proceeds following standard paired-end sequencing protocol. Index 1 and Index 2 primers and sequences are shown in part (B). Dual indexing comprises the addition of a unique Index 1 (i7) and a complementary unique Index 2 (i5) to each sample from a selection of 12 Index 1 adaptors and 8 index 2 adapters. The eight base Index 1 (i7) indices are adjacent to the P7 sequence and the eight base Index 2 (i5) are adjacent to the P5 sequence.

Briefly, 200 µl of 80% (v/v) ethanol was added to each sample well ensuring that the beads were covered. After 30 s the supernatant was removed and discarded and the beads were air dried for 15 min. Following this, the 96 well plate was removed from the magnetic stand and 52.5 µl of Resuspension Buffer (Illumina Nextera Kit) was added to each sample well and mixed by pipetting up and down ten times. Samples were incubated at room temperature for two min and placed on a magnetic stand until the supernatant cleared. A 50 µl aliquot of supernatant containing the cleaned PCR product was then transferred to a new PCR tube for each sample.

6.2.4.4 Library normalisation and pooling

The concentration of double-stranded DNA was determined for each sample using the Qubit 2.0 Fluorometer and the Qubit double stranded high sensitivity assay kit (Life technologies). Once determined, the concentration of each sample was normalised by transferring the volume of each sample required for an equal concentration per sample in the final pooled solution in one pooled sample Eppendorf tube. An equal volume of 0.2 M sodium hydroxide (NaOH) was added to the pooled sample to give a final concentration of 0.1 N NaOH and this was incubated for 5 min at room temperature to denature the double-stranded DNA. This was followed by the addition of an equal volume of LNS1 buffer, supplied with the Nextera XT kit (Illumina).

6.2.4.5 Quality assessment and final measurement of prepared DNA quality prior to WGS

The quality and final concentration of the denatured pooled library was checked using the Kapa qPCR library quantification kit (Kapa Biosystems, 20-22 Bedford row, London, UK). The library concentration was used to calculate the volume of the library required within 600 µl volume of HT1 buffer, supplied with the Nextera XT kit (Illumina) to give a final DNA concentration of 12.5-15 pM. The MiSeq reagent cartridge was allowed to thaw slowly at room temperature. The pooled library diluted in HT1 buffer was incubated at 96°C for two min, followed by inversion twice and it was then placed in an ice water bath for five min. The diluted amplicon library was loaded into the MiSeq reagent cartridge and the library was sequenced using a MiSeq desktop sequencer (illumina) according to the manufacturer instructions.

6.2.4.6 Sequencing, assembly and analysis

Reads were downloaded as fastQ files from the MiSeq and uploaded into the BioNumerics genome analysis tools (GAT) software, version 7.5, (Applied Maths). Paired-end reads were checked for quality, trimmed and assembled by resequencing using a reference ST22-MRSA-IV strain, HO 5096 0412 (Holden *et al.*, 2013), as a scaffold for the seven ST22-MRSA-IV query strains. Chromosomal comparisons were performed using BioNumerics by aligning the seven isolates investigated in this study against the genome sequence of an ST22-MRSA-IV strain (M07/0319) investigated as part of another study in this laboratory (Unpublished data) and the number of SNVs identified per isolate when aligned against M07/0319 was subsequently compared with all other isolates. The resultant SNV data was then analysed using the BioNumerics GAT module.

The *icaC* gene sequence of the seven ST22-MRSA-IV isolates was also investigated using the whole-genome sequences. A multiple sequence alignment was performed using ClustalW2 (Larkin *et al.*, 2007) to compare the *icaC* sequences of the seven ST22-MRSA-IV isolates against *icaC* from reference *S. aureus* strains obtained from Genbank including COL (CP00046.1), Newman (AP009351.1), MRSA252 (BX571856.1) and a ST22-MRSA reference strain HO 5096 0412 (HE681097).

6.3 Results

6.3.1 DNA microarray analysis of ST22-MRSA-IV isolates recovered from patients with BSIs in Irish hospitals in 2006/07 and 2009.

In order to determine if the lack of detection of *icaC* in ST22-MRSA-IV isolates recovered in 2011 compared to 2006 was also apparent an additional 24 and 24 ST22-MRSA-IV isolates recovered in 2006 and 2009/10, respectively, were investigated by DNA microarray profiling. The virulence and antimicrobial resistance genes detected among these isolates are shown in Figure 6.3. All isolates harboured *blaZ*, *egc*, *clfA* & *clfB*, *cna* and *fnbA* while carriage of *erm(C)* (58.3%, 14/24, 2006; 62.5%, 15/24, 2009/10), *bbp* (100%, 24/24, 2006; 95.8%, 23/24, 2009/10) and *sec* & *sel* (58.3%, 14/24, 2006; 37.5%, 9/24, 2009/10) were variable. The IEC genes were present in almost all isolates (91.7%, 22/24, 2006; 100%, 24/24, 2009/10). The majority of isolates in both study periods harboured IEC type B (*sak*, *chp* & *scn*) (100%, 22/22, 2006; 95.8%, 23/24, 2009/10) with one isolate from 2009/10 carrying IEC type E (*sak* & *scn*). In 2006, 75% (18/24) of the isolates were found to harbour the three genes of the *ica* cluster, namely *icaA*, *icaC* and *icaD*, while in the remaining 25% (6/24) of isolates only *icaA* and *icaD* were detected. In 2009/10 the three *ica* genes were only detected in 25% (6/24) of isolates with the majority of isolates (75%, 18/24) carrying *icaA* and *icaD* only.

6.3.1.1 icaC sequence analysis

Whole-genome sequencing of seven ST22-MRSA-IV isolates, four from 2006/07 and three from 2011 (Table 6.1) revealed the presence of *icaC* in all isolates, despite the fact that two of the three isolates from 2011 (R058 and R030) yielding negative and ambiguous results, respectively, for *icaC* by DNA microarray profiling. The other eight isolates were *icaC* positive by microarray profiling. Further analysis of the *icaC* gene within each isolate's WGS was undertaken to determine if nucleotide sequence variation, and therefore potential lack of array primer or probe binding, was the reason for the lower rate of detection of *icaC* by the DNA microarray in isolates from BSIs in 2009/10 and 2011 compared to 2006. The comparative sequence analysis revealed the presence of identical *icaC* gene sequences in all seven ST22-MRSA-IV isolates. A multiple sequence alignment compared the *icaC* sequences of the seven ST22-MRSA-IV isolates investigated in the present study to several *icaC* sequences from reference *S. aureus*

strains from Genbank (Fig. 6.4). There were 12 differences detected which were unique to the ST22-MRSA-IV isolates, including the reference ST22-MRSA-IV strain HO 5096 0412 and the seven isolates subjected to WGS in the present study (Fig. 6.4). This included a T instead of C at position 721 which was found to be located within the binding region of the *icaC* primer for the DNA microarray (Fig. 6.4).

6.3.2 Comparative growth rate analysis of ST22-MRSA-IV isolates recovered from patients with BSIs in Irish hospitals in 2006, 2009 and 2011.

Figure 6.5 shows the results of growth curves for the three groups of ST22-MRSA-IV investigated over a 24-hour period. Group A consisted of six isolates from 2006 and six from 2011, group B contained five isolates each from 2006 and 2011 and group C contained 12 isolates six each from 2006 and 2009/10 (Table 6.1). Statistically significant differences in the growth rates of ST22-MRSA-IV isolates were identified at time points between six and eight hours (6 hours $p=0.020305$; 7 hours $p=0.0089$; 8 hours $p=0.01875$) for two groups (Group A and B) of ST22-MRSA-IV isolates from 2006 and 2011 (Fig. 6.5 (a) & (b)), with isolates from 2011 growing slower than those from 2006. All isolates reached a similar level of growth by 24 h. Although differences were identified in the growth rates of ST22-MRSA-IV isolates from 2006 and 2009 (Group C), these were not statistically significant (Fig. 6.5 (c)) Overall, the ST22-MRSA-IV isolates from 2006 grew faster than those recovered in 2009 and even more so than those recovered in 2011.

6.3.3 Whole-genome sequence analysis of four of the fastest growing isolates from 2006 and three of the slowest growing isolates from 2011, to identify any SNVs that may have contributed to the possible clonal decline of ST22-MRSA-IV.

The number of SNVs identified by pairwise comparison of all isolates after alignment to a ST22-MRSA-IV isolate (M07/0319) previously analysed by WGS in this laboratory, are shown in Table 6.2. Overall, an average SNV difference of 82.5 was identified between ST22-MRSA-IV isolates from 2006 and 2011 (standard deviation (SD) 56.86), with SNV differences ranging from nine (between R017 (2011) and R022 (2006) and between R005 (2006) and R016 (2006)) to 183 (between R058 (2011) and R005 (2006)).

Within the 2006 isolates there was an average SNV difference of 83.6 (SD 54.46) between isolates, ranging from nine to 136. There were slightly less SNV differences between the 2011 isolates with an average of 59.3 (SD 28.57), ranging from 32 to 89.

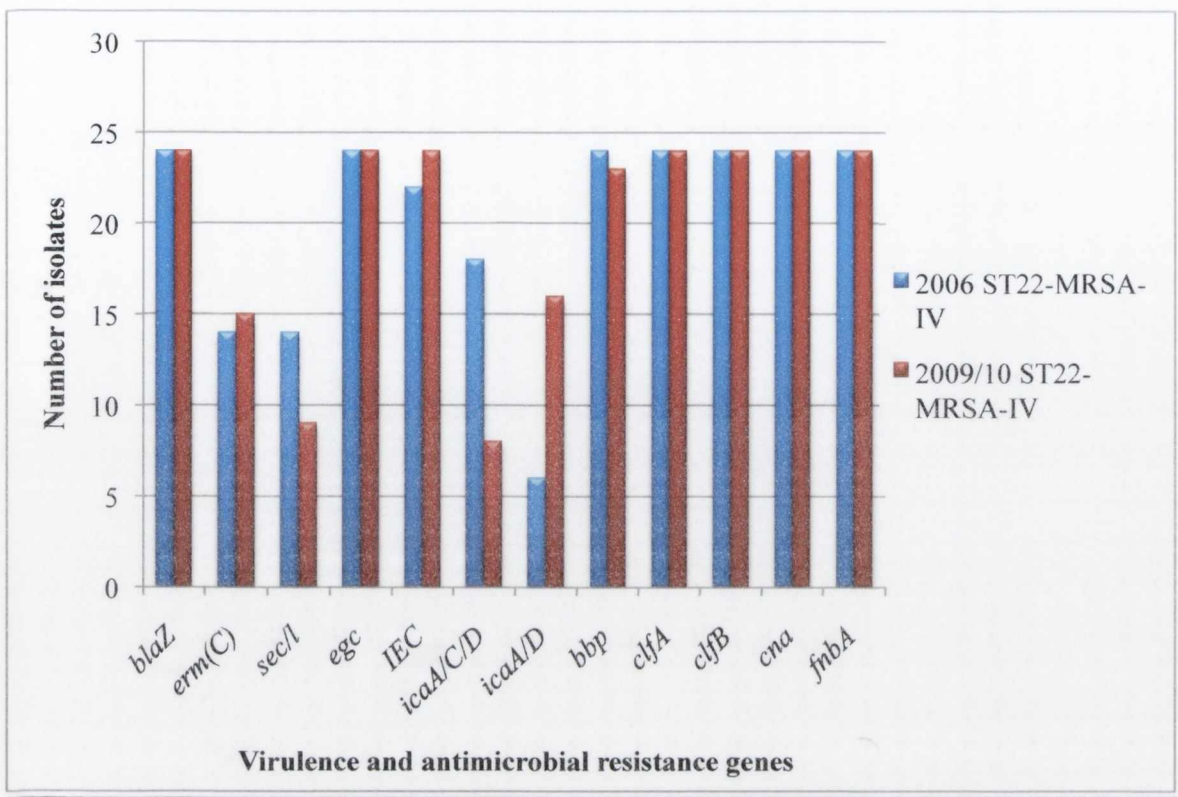


Figure 6.3 Numbers of ST22-MRSA-IV isolates recovered from patients with BSIs in Irish hospitals in 2006 or 2009/10 harbouring each of the genes listed as determined by DNA microarray profiling. *icaA/C/D* includes all the isolates which had the full combination of the *ica* locus genes and *icaA/D* includes those isolates which contain *icaA* and *icaD* but not *icaC*.

R016 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
HO ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R005 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R017 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R022 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R030 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R058 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
R060 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
MRSA252 ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCCATTATATGTGCAATTATTATT 60
COL_ ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60
Newman ATGAAAAAGATTAGACTTGAACCTCGTATATTTACGTGCTATTATATGTGCAATTATTATT 60

R016 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
HO GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R005 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R017 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R022 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R030 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R058 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
R060 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
MRSA252 GTTACACATTTATTAACACAAATTACTTTAAAACATGAGAATATGGAAGGAGGATCTTTA 120
COL_ ATCACACATTTACTTACACAAATTACTTTAAAACATGAGAATATGGAAGGATCTTTA 120
Newman ATCACACATTTACTTACACAAATTACTTTAAAACATGAGAATATGGAAGGATCTTTA 120
* *****

R016 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
HO GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R005 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R017 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R022 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R030 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R058 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
R060 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCATGTTTTATTATTTTG 180
MRSA252 GTACTACAGTTCTATATTCGTAATATTGTCATTTTTGGTACACCTTGCTTTATTATCTTG 180
COL_ GTGTTACAATTTTACATTTCGTAATATTGTGATTTTTGGTACACCTTGCTTTATTATCTTG 180
Newman GTGTTACAATTTTACATTTCGTAATATTGTGATTTTTGGTACACCTTGCTTTATTATCTTG 180
** *****

R016 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
HO TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R005 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R017 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R022 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R030 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R058 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
R060 TCGCAGTTATTGACGACTTTAAACTATCAAAAAGTCACCTATAGATACTTAACTACACGC 240
MRSA252 TCACAGTTACTGACAACCTTGAATTACCAAAAAGTCACCTATAGATACTTAACTACACGC 240
COL_ TCACAGTTACTGACAACCTTGAATTACCAAAAAGTCACCTATAGATACTTAACTACACGC 240
Newman TCACAGTTACTGACAACCTTGAATTACCAAAAAGTCACCTATAGATACTTAACTACACGC 240
** *****

R016 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
HO GTAAAAATATATACTTATTCCTTACATATTAATGGGAT-GTTTTACAGTTATAGTGAATCA 299
R005 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
R017 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
R022 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
R030 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
R058 GTAAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300

R060 GTAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
MRSA252 GTAAAATATATATTGATTCCCTTACATATTAATGGGTTTGTGTTTTACAGTTATAGTGAATCA 300
COL_ GTAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
Newman GTAAAATATATACTTATTCCTTACATATTAATGGGATTGTTTTACAGTTATAGTGAATCA 300
***** * *****

R016 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
HO TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 359
R005 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
R017 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
R022 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
R030 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
R058 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
R060 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
MRSA252 TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
COL_ TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
Newman TTATTAACAGATTCAAGTTTCAATAAAACAATTCATTGAAAATGTCCTATTAGGTCAATGG 360
***** ** *****

R016 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
HO ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 479
R005 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
R017 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
R022 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
R030 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
R058 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
R060 ATTAACCTATAACCTATTCAATAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
MRSA252 ATTAACCTATAACCTATTCAACAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
COL_ ATTAACCTATAACCTATTCAACAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
Newman ATTAACCTATAACCTATTCAACAGTAAAATATTATTATTGTTATCTTTTATTTTACAGCAA 480
***** *****

R016 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
HO CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 599
R005 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
R017 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
R022 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
R030 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
R058 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
R060 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
MRSA252 CCATTAAGTGAAAACACTATAATATTCGGATGGATTTTTTATTTCTTTTCTTAGGTGCATAT 600
COL_ CCATTAAGTGAAAATACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
Newman CCATTAAGTGAAAATACTATAATATTCGGATGGATTTTTTATTTCTTCTTAGGTGCATAT 600
***** *****

R016 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
HO ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 659
R005 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
R017 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
R022 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
R030 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
R058 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
R060 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
MRSA252 ATGGGTTATAACTACGAACGTGTTTTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
COL_ ATGGGTTATAACTACGAACGTGATTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
Newman ATGGGTTATAACTACGAACGTGATTAAATTTCTTAGAACGTTATTTAGTTATTATGATT 660
***** *****

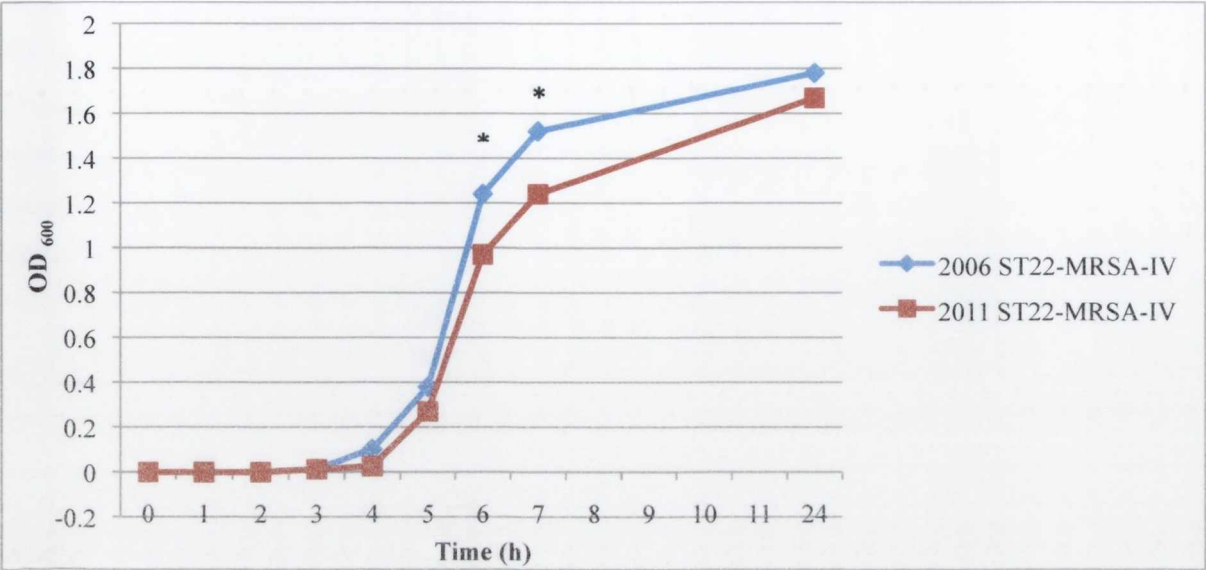
R016 TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 756
HO TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 755
R005 TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 756
R017 TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 756
R022 TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 756
R030 TTAGCAAATGGAGACTATTGGAATGTTACCAGCTTTTCATATTCATTAACACCATATAA 756



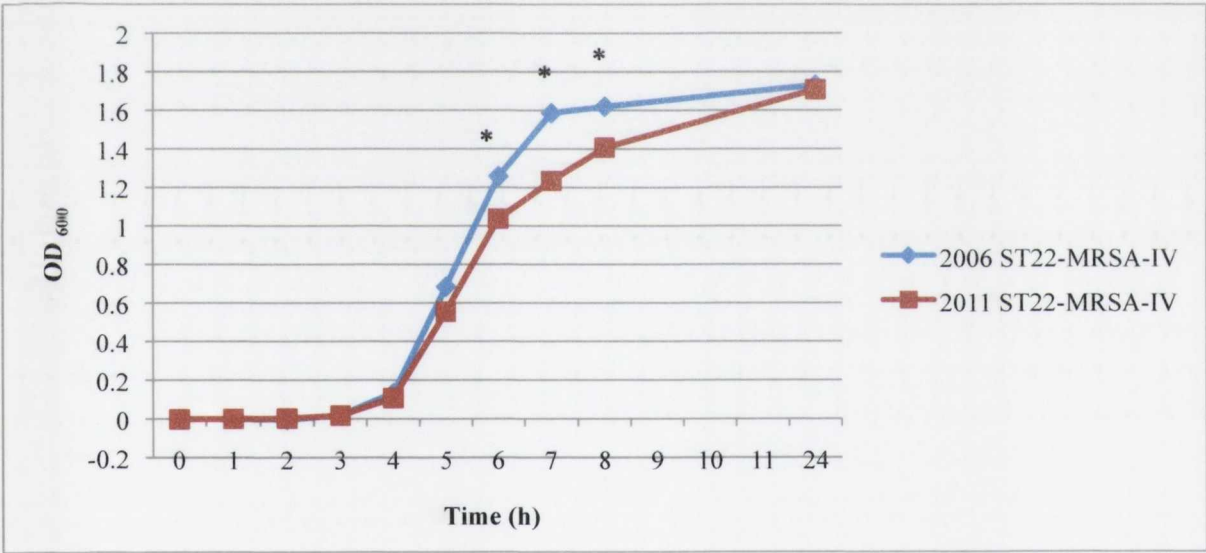
Figure 6.4 Multiple sequence alignment of the seven *icaC* sequences from the ST22-MRSA-IV isolates identified as part of the present study by whole-genome sequencing (R005, R016, R017, R022, R030, R058 and R060), a reference ST22-MRSA-IV strain HO50960412 (abbreviated to HO in figure; Genbank accession no. HE681097) and reference *S. aureus* strain *icaC* sequences from strains COL (CP000046.1), Newman (AP009351.1) and MRSA252 (BX571856.1).

Asterisk (*) indicate sequence homology between the strains. Nucleotide bases indicated in red are the DNA microarray *icaC* primer sequence. Nucleotide bases highlighted with yellow indicate the variation identified between the *icaC* sequence in the ST22-MRSA-IV isolates and the DNA microarray primer sequence for *icaC* on the DNA microarray; the ST22-MRSA-IV isolates investigated in the present study all encoded a nucleotide difference of a thymine (T) instead of a cytosine (C) within the primer binding site.

(a)



(b)



(c)

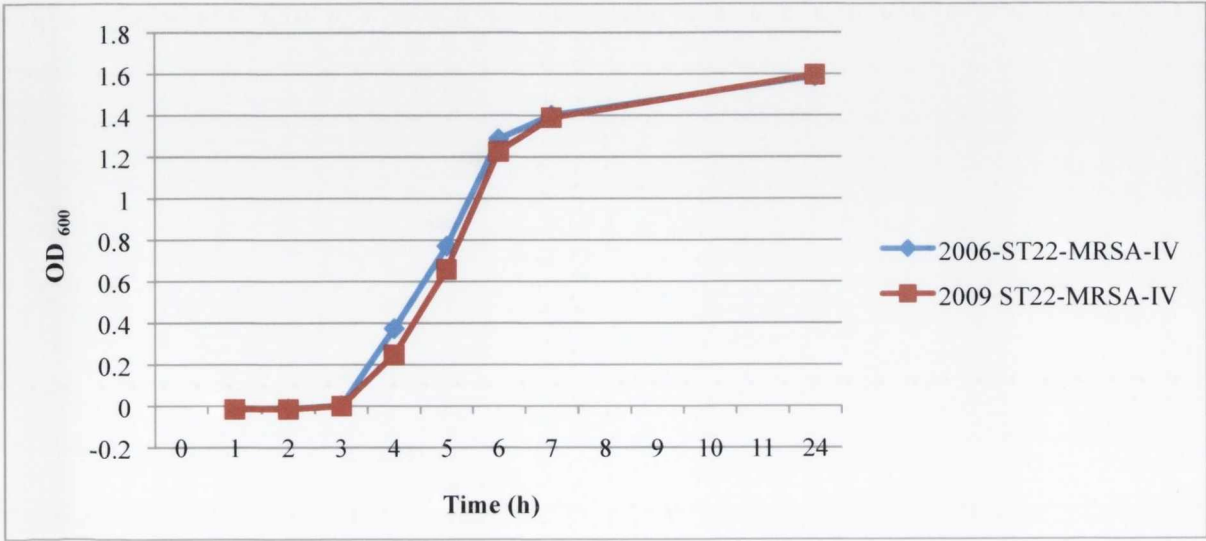


Figure 6.5 Trendline graphs of three replicates of growth curves of ST22-MRSA-IV isolates recovered from patients with bloodstream infections in Irish hospitals in 2006, 2009 and 2011. (a) Six isolates from 2006 and six isolates from 2011 (Group A); (b) Five isolates from 2006 and five isolates from 2009 (Group B); (c) Six isolates from 2006 and six isolates 2009 (Group C). Isolates were grown for 24 h at 37°C in Brain Heart Infusion broth and the optical density at 600 nm was measured hourly for the first eight hours and again at 24 h. Timepoints at which there was a statistically significant difference between groups of isolates are indicated with an asterisk (*).

Table 6.2 Matrix showing the number of single nucleotide variations (SNVs) identified in the seven ST22-MRSA-IV isolates recovered in the present study from patients with bloodstream infections in Ireland in 2006 and 2011

		No. of SNV differences						
Year		2006				2011		
	Isolate no.	R005	R016	R022	R060	R017	R030	R058
2006	R005							
	R016	9						
	R022	103	94					
	R060	136	127	33				
	R017	94	85	9	42			
2011	R030	126	117	23	10	32		
	R058	183	174	80	47	89	57	

¹Isolates were compared and aligned to a previously described ST22-MRSA-IV isolate M07/0319 investigated previously by this laboratory (unpublished data). All SNVs were included provided they were detected with a coverage >40.

Some of the 2006 isolates exhibited greater SNV differences to each other than to 2011 isolates. Similarly some of the 2011 isolates had greater SNV differences with each other than when compared to the 2006.

When the SNVs that were identified among the 2006 and 2011 isolates by alignment to the reference ST22-MRSA-IV isolate (M07/0319) were compared with each other, there were amino acid changes identified that resulted in missense mutations in genes among some or all isolates in one study period which were not detected among those from the other study period. In addition, some amino acid changes resulting in missense mutations were detected in isolates from both study periods. All of these amino acid changes are described in detail below.

6.3.3.1 Genes harbouring missense mutations which were found uniquely in some or all of the 2006 isolates

Overall, unique missense mutations were detected in the 2006 isolates in proteins involved in several different functions such as biofilm/binding including extracellular matrix protein and fibronectin binding protein A, antimicrobial resistance such as the arsenical resistance operon repressor protein and penicillin binding protein 2, structural components such as teichoic acid biosynthesis protein and various proteins involved in metabolism, virulence and biosynthesis pathways (Table 6.3).

Fifty-two genes uniquely harboured 83 missense mutations in the four 2006 ST22-MRSA-IV isolates (Table 6.3). However, the missense mutations identified within 45/52 of these genes were detected in only one of the four 2006 isolates (Table 6.3).

Five genes harboured missense mutations which were detected in two isolates each. In three of these genes the same missense mutation was identified in both isolates. This included (i) three lysine (K) to serine (S) amino acid transitions and a methionine (M) to isoleucine (I) transition in a putative cobalamin synthesis protein in isolates R022 and R060, (ii) phenylalanine (F) and arginine (R) to K, and F and R to S in the ferrichrome binding protein precursor in both R022 and R060 and (iii) alanine (A) to S, valine (V) to I and three tyrosine (Y) to I amino acid transitions in the arsenical resistance operon repressor 2 in both R016 and R005 (Table 6.3).

Missense mutations were identified in two genes each in three of the four isolates. This included the extracellular matrix binding protein Ebh and the hyaluronate lysase precursor protein, although each isolate had a different mutation (Table 6.3).

6.3.3.2 Genes harbouring missense mutations which were found uniquely in some or all of the 2011 isolates

Unique missense mutations were detected in proteins in the three 2011 isolates which were associated with biosynthesis e.g. shikimate 5-dehydrogenase, metabolism, replication, binding and biofilm e.g. collagen adhesion precursor and virulence, amongst others (Table 6.4).

The 2011 ST22-MRSA-IV isolates harboured a total of 44 unique missense mutations in 40 genes but only one of these genes contained a missense mutation detected in >1 isolate i.e. the glucose specific transporter protein IIABC which exhibited a F to leucine (L) amino acid transition in R017 and a glycine (G) to S amino acid transition in R030. No gene was found to harbour missense mutations common to all three 2011 isolates (Table 6.4).

6.3.3.3 Genes harbouring missense mutations which were identified in isolates from both the 2006 and 2011 isolates

Proteins in which missense mutations were detected in one or more isolates from both study periods had functions similar to those which were unique to the 2006 or 2011 groups i.e. biosynthesis e.g. uracil phosphoribosyltransferase, antimicrobial resistance e.g. *arsC*, binding and biofilm e.g. extracellular matrix protein A and the bone sialoprotein binding protein and others such as metabolism, cell division transport and virulence (Table 6.5).

A total of 47 genes were identified which exhibited 348 missense mutations in isolates from both 2006 and 2011 (Table 6.5). Eighteen of these 47 genes were represented by a single isolate in each year (Table 6.5). The same amino acid transition was identified for 14 of these 18 genes in isolates from both study periods, and two isolates (R060, 2006 and R030, 2011) accounted for 12 of these changes. Isolates R016 (2006) and R030 (2011) harboured the same mutations in 15 of the 47 genes, including uracil phosphoribosyl transferase (M to Threonine (T) amino acid change) and topoisomerase IV subunit B (Proline (P) to S amino acid change). Isolates R060 (2006)

Table 6.3 Proteins encoded by genes harbouring missense mutations identified in the ST22-MRSA-IV isolates recovered from patients with BSI in Ireland, which were unique to the 2006 isolates

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²			
		R016	R005	R022	R060
Acetyltransferase	Acetyltransferase, GNAT family protein ³	F-984278-S	ND ⁴	ND	ND
Biofilm/binding	AraC family regulatory protein Extracellular matrix binding protein B	L-1562245-F ND	ND	ND	ND
			D-1429749-E, N-1429634-A, N-1429718-D, N-1429714,1429715-R, N-	ND	ND
			1429737,1429738-T, S-1429773,1429775-G, S-	ND	ND
			1429752,1429754-T, T-	ND	ND
Antimicrobial resistance	Fibronectin-binding protein FnbA MHC class II analog Arsenical resistance operon repressor 2	ND	1429726,1429776,1429777-N, T-1429636-Q	V-2566808-I	ND
			ND	ND	ND
			T-2007470-S A-1837932-S, V-1837947-I, Y-1837938,1837939,1837940-I	ND	ND
			ND	ND	ND
Peptidase	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein Penicillin-binding protein PBP2 TetR family regulatory protein Cytosol aminopeptidase family	Q-2573344-H ND V-2609063-L ND	ND	ND	ND
			ND	ND	ND
			ND	ND	ND
			ND	ND	ND

Continued Overleaf

A-1466054-V
ND
G-863419-*

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²			
		R016	R005	R022	R060
Protein synthesis	DNA-directed RNA polymerase delta subunit	ND	ND	D-2182972-E, L-2182975,2182976,2182977-D	ND
Virulence	Gamma-hemolysin component C precursor	ND	ND	ND	W-2478515-L
Phage	Phage dUTPase	N-1532065,1532067-Y, SE-1532477-NE, V-1532071,1532072-E	ND	ND	ND
Information pathways Structural	Phage membrane protein	LT-1531429-QT, V-1531369-A	ND	ND	ND
	Sensor kinase protein	ND	I-2130114-V	ND	ND
	Teichoic acid biosynthesis protein	ND	ND	ND	L-650326-P
Metabolism	Formate-tetrahydrofolate ligase	V-1785049-X	ND	ND	ND
	Hyaluronate lyase precursor 2	S-2254726-G	ND	V-2268569-M	T-2273158-I
	Methionyl-tRNA formyltransferase	ND	ND	ND	T-1156778-A
	Nitrate reductase alpha chain	S-2435094-I	ND	ND	ND
	Phosphoenolpyruvate carboxykinase	ND	ND	ND	F-1866862-S
	Phosphoenolpyruvate-protein phosphotransferase	ND	ND	ND	A-1019099-S
	Sucrose operon repressor	ND	G-2082115-S	ND	M-2092376-I
	Threonyl-tRNA synthetase	ND	ND	ND	D-1731543-G
	Urease accessory protein UreF	G-2321927-D	ND	ND	ND
	Zinc metalloproteinase aureolysin precursor	ND	ND	ND	A-2724477-T
	Haloacid dehalogenase-like hydrolase	ND	ND	ND	G-2369575-S
	Putative 30S ribosomal protein S1	ND	ND	ND	Q-1493457-H

Continued Overleaf

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²			
		R016	R005	R022	R060
Putative acyl-CoA dehydrogenase		ND	G-238063-V	ND	ND
Putative bifunctional purine Biosynthesis protein [includes: Phosphoribosylaminoimidazole carboxamide		ND	D-1006255-N	ND	ND
Putative biotin carboxylase subunit of acetyl-CoA carboxylase		ND	D-1656170-G	ND	ND
Putative branched-chain amino acid transport system carrier protein		ND	Q-182424-H	ND	ND
putative carbon-nitrogen hydrolase		A-2066558-S	ND	ND	ND
Putative choloylglycine hydrolase		E-289275-K	ND	ND	ND
Putative cobalamin synthesis protein		ND	ND	K-697516,697517,697518-S, M-697515-I	K-701227,701228,701229-S, M-701226-I
Putative CTP synthase		ND	P-2173868-L	ND	ND
Putative DNA topoisomerase		ND	M-2295569-T	ND	ND
Putative exodeoxyribonuclease VII large subunit		ND	M-1583711-T	ND	ND
Putative ferrichrome-binding protein precursor		ND	ND	FR-2069937-K, FR-2069939-S, I-2070594-T	FR-2073607-K, FR-2073609-S
Putative glycyl-tRNA synthetase		H-1616573-R	ND	ND	ND
Putative hydrolase		D-2637690-N	ND	ND	ND
Putative L-serine dehydratase, alpha chain		ND	ND	G-2597966-C	ND
Putative malolactic enzyme		L-789728-V	ND	ND	ND
Putative oligopeptide transporter ATPase		ND	V-1360856-I	ND	ND

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²			
		R016	R005	R022	R060
	Putative peptidase	ND	V-556332-I	A-1365873-V	ND
	Putative phage regulatory protein	ND	ND	G-2051427,2051428-SQ, I-2051417-GM, N-2051416-GD	ND
	Putative radical activating enzyme	ND	D-722204-N	ND	ND
	Putative recombination protein U	D-1457398-H	ND	ND	ND
	Putative solute binding lipoprotein	D-2445017-G	ND	ND	ND
	Putative zinc-binding dehydrogenase	ND	ND	F-2253947-V	ND
	Tn554-related, transposase B	ND	ND	ND	Q-2006037-*
	Transposase	L-2782625-F	ND	ND	ND

¹Missense mutations with a coverage <40 in the whole-genome sequencing analysis were excluded from the analysis.

²Amino acid transitions are indicated with the first letter indicating the amino acid encoded by the ST22-MRSA-IV reference strain M07/0319. The number indicates the nucleotide position within the whole-genome sequence of the 2006 isolate indicated and the final letter indicates the amino acid encoded by the 2006 isolate.

Abbreviations: A, Alanine; B, Aspartic acid or Asparagine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; X, unspecified or unknown; Y, Tyrosine; Z, Glutamine or Glutamic acid; (*) Insertion of a new stop codon.

³Proteins highlighted in red font have been found previously to be essential to *S. aureus* growth in BHI in other studies (Forsyth *et al.*, 2002; Valentino *et al.*, 2014).

⁴ND, None detected.

Table 6.4 Proteins encoded by genes harbouring missense mutations identified following whole genome sequencing of three ST22-MRSA-IV isolates recovered from patients with BSIs in Ireland, which were unique to the 2011 group

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transition ²		
		R017	R030	R058
Biosynthesis	2-isopropylmalate synthase	G-2094712-S	ND ³	ND
	Cytidylate kinase ⁴	ND	M-1494220-T	ND
	Deoxyribose-phosphate aldolase	V-124288-I	ND	ND
	Dihydrodipicolinate reductase	ND	D-1381515-N	ND
	S4 domain containing protein	AT-499084-T	ND	ND
Metabolism	Shikimate 5-dehydrogenase	S-1642195-G	ND	ND
	arginase	ND	L-2229125-Q	ND
	Cysteine protease precursor	L-972166-F	ND	ND
	NADH-dependent FMN reductase	ND	A-358192-V	ND
	Nitrite reductase large subunit	T-2440688-I	ND	ND
DNA Replication	Tagatose 1,6-diphosphate aldolase	ND	E-2263339-K	ND
	Urease beta subunit	ND	ND	A-2182224-T
	Chromosome replication initiation/membrane attachment protein ³	R-1724454-C	ND	ND
	Collagen adhesin precursor	ND	H-2785737-R	ND
	RGD-containing lipoprotein SA2447	P-205041-S I-2735028-T, S-2733558-F	ND ND	ND
Transport	Glucose-specific PTS transporter protein, IIABC component ⁵	F-186640-L	G-189143-S	ND
Regulation	Preprotein translocase SecA subunit-like protein	ND	ND	A-2573489-V
	OsmC-like protein	ND	V-1779551-A	ND
	Topoisomerase IV subunit A	M-1314570-T	ND	ND

Continued Overleaf

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transition ²	
		R030	R058
Virulence	Truncated beta-hemplysin	AM-2052274-T	ND
Defense	Type I restriction modification system modification protein	ND	ND
Other/unknown	Putative acetolactate synthase	ND	ND
	Putative cardiolipin synthase	ND	ND
	Putative cobalamin synthesis protein	ND	ND
	Putative copper importing ATPase A	ND	ND
	Putative deaminase	ND	ND
	Putative dioxygenase	ND	ND
	Putative dUTP pyrophosphatase	ND	ND
	Putative endonuclease	ND	ND
	Putative GMP synthase	ND	ND
	Putative metalloregulator	ND	ND
	Putative permease	ND	ND
	Putative phosphoribosylaminoimidazole carboxylase ATPase subunit	ND	ND
	Putative phosphoribosylformylglycinamide synthase I	ND	ND
	Putative phytoene dehydrogenase related protein	ND	ND
	Putative pyrimidine-nucleoside phosphorylase	ND	ND
	Putative short chain dehydrogenase	ND	ND
	Putative tagatose-bisphosphate aldolase	ND	ND

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transition ²	
		R017	R030
	Putative transcription-repair coupling factor	ND	ND
			W-478979-*

¹ Missense mutations with a coverage <40 in the whole-genome sequencing analysis were excluded from the analysis.

² Amino acid transitions are indicated with the first letter indicating the amino acid encoded by the ST22-MRSA-IV reference strain M07/0319. The number indicates the nucleotide position within the whole-genome sequence of the 2006 isolate indicated and the final letter indicates the amino acid encoded by the 2006 isolate.

Abbreviations: A, Alanine; B, Aspartic acid or Asparagine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; X, unspecified or unknown; Y, Tyrosine; Z, Glutamine or Glutamic acid. (*) Insertion of a new stop codon.

³ND, none detected.

⁴Proteins highlighted in red font have been found previously to be essential to *S. aureus* growth in BHI in other studies (Forsyth *et al.*, 2002; Valentino *et al.*, 2014).

⁵The protein highlighted in italics was the only protein found to contain missense mutations in more than one of the 2011 isolates investigated.

Table 6.5 Proteins encoded by genes harbouring missense mutations which were common to ST22-MRSA-IV isolates from 2006 and 2011

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²						
		2006			2011			
		R005	R016	R022	R060	R017	R030	R058
Biosynthesis	3-isopropylmalate dehydratase large subunit	ND ³	ND	E-2109831-K	ND	ND	A-2112762-D	ND
	Translation elongation factor G	ND	ND	L-552306,552307-K	ND	L-551200,551201-K	L-534807,534808-K	ND
	Uracil phosphoribosyl-transferase	M-2159788-T	M-2152220-T	M-2167031-T	M-2170699-T	M-2154390-T	M-2169676-T	M-2020033-T
2	Arsenical pump membrane protein	D-1842489-G, F-1841449-L, I-	D-1839414-G, F-1838374-L, I-1839479,1839481-V, K-	D-1845131-G, I-1845196,1845198-V, L-1845031,1845033-M, T-1845173-I	ND	D-1837496-G, I-1837399,1837561,1837563-V, L-1837396,1837398-M, T-1837538-I	ND	ND
		I-1842554,1842556-V, S-	81-V, K-	98-V, L-				
		1841492,1841494-A, T-1842531-I	1838447-E, S-1838417,1838419-A, T-1839456-I					

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²					
		2006			2011		
		R005	R016	R022	R060	R017	R030 R058
Binding / Biofilm	Bone sialoprotein-binding protein	V-577470-A	ND	V-576058-A	V-579772-A	G-576352-R, V-574952-A	V-579246-A V-557589-A
	Capsular polysaccharide synthesis enzyme	ND	ND	ND	A-140679-E	ND	A-140435-E ND
	Extracellular matrix binding protein	*INDEL	R-1412851-H, T-1419401-A	ND	ND	ND	A-1445056-T, A-1432002-V, G-1432543-S ND
	EbhA	ND	ND	A-748036-T	ND	A-746540-T	ND
	FecCD transport family protein	ND	D-574939-E	ND	V-576057-I	E-571636-K	V-575531-I ND
	LPXTG surface protein	ND	ND	ND	V-378312-A	ND	V-377993-A ND
Metabolism	Cys/Met metabolism PLP-dependent enzyme	T-1287797-A	T-1284920-A	T-1287995-A	T-1291658-A	T-1282733-A	T-1291012-A ND
	Homoserine kinase	F-2759581-S, H-2758424-Y	ND	F-2768014-S	F-2770285-S	F-2752610-S	F-2769068-S ND
	Lipase precursor	ND	ND	ND	RE-397102-*	ND	RE-396783-* ND
	Nitroreductase family protein	ND	A-2480657-P	ND	ND	R-2484672-L	ND
Cell Division	Sodium/hydrogen exchanger family protein	ND					
	FtsK/SpoIIIE family protein	D-1800588-E	D-1797510-E, T-1799092-I	D-1803224-E	D-1806858E	D-1795732-E	D-1806156-E ND

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²				
		2006		2011		
		R005	R016	R022	R060	R017 R030 R058
Transport	ABC transporter ATP-binding protein ⁴	I-2087378-V	I-2079799-V, N-1007534-I, P-695166-L, T-446668-I	I-2094539-V, N-1008686-I	E-2707931-K, I-2098208-V, N-1012354-I	A-2393785-V, I-2081958-V, N-1005693-I E-2706714-K, I-2097251-V, N-1011786-I ND
	Glycine betaine transporter 2	S-2244161-A	S-2236588-A	S-2251412-A	S-2255091-A	S-2238749-A S-2254035-A ND
	Truncated transposase	A-2474189-T	GK-1975181-K, S1975127-K	ND	ND	A-2469222-T, E-2469235,2469237-Q ND ND
DNA replication	DNA repair protein RadC	I-1702252-T	I-1699203-T	I-1704764-T	I-1708398-T	I-1697540-T I-1707696-T ND
	Holliday junction DNA helicase	R-1694750-H	ND	R-1697262-H	R-1700896-H	R-1690038-H R-1700194-H ND
	Topoisomerase IV subunit B	P-1317035-S	P-1313639-S	D-1317291-V, P-1317305-S	P-1320968-S	D-1311793-V, P-1311807-S P-1320322-S ND
Phage	Hypothetical phage protein	*-2041810-Q, D-1530704-Y	ND	*-2048926-Q	*-2052574-Q	*-2036694-Q, P-2040821-S P-1888236-Q, T-1894405,1894407-A ND ND ND
Virulence Other / Unknown	Exotoxin Autolysin Nucleoside permease Probable tRNA modification GTPase	ND ND ND	ND ND ND	ND H-2023436-Y	H-415749-Y H-2027073-Y V-525670-I	H-415430-Y H-2026211-Y V-525144-I K-2804885-R K-2636014-R

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²					
		2006			2011		
		R005	R016	R022	R060	R017	R030
Putative 6-pyruvoyl tetrahydropterin synthase Putative ABC transporter ATP-binding protein Putative ATP synthase subunit b Putative enterotoxin Putative exported protein	ND	ND	T-721603-K	ND	T-720107-K	ND	ND
	I-292048-R	ND	ND	ND	S-160485-F	ND	ND
	L-2156048-S	ND	L-2163291-S	L-2166959-S	L-2150650-S	L-2165936-S	ND
	ND	ND	C-1653326-Y	C-1656928-Y	C-1646079-Y	C-1656226-Y	ND
	F-1455890-L, G-2336220, 2336221-Y, S-2336226, 2336227, 2336228-Y, Y-2336223, 2336225, 2336229-N	ND	R-2020298-G	R-2023935-G	V-2263013-I	R-2023073-G	ND
Putative glucose inhibited division protein B Putative glucosyl transferase Putative lipoprotein	ND	ND	ND	D-2802622-N	ND	D-2801404-N	ND
	ND	ND	Y-715814-H	Y-719538-H	Y-714318-H	Y-718979-H	ND
	V-1550628-I	S-80412-P	L-424819-S, S-83140-P	S-82999-P	ND	D-1153731-G, S-82755-P	V-1092487-A

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²						
		2006			2011			
		R005	R016	R022	R060	R017	R030	R058
	Putative Na ⁺ dependent nucleoside transporter	ND	ND	ND	I-659365-V	ND	I-658806-V	ND
	Putative NADH-Ubiquinone/plastoquinone (complex I) protein	ND	A-435617-P	ND	ND	F-434143-L	ND	ND
	Putative permease protein	D-2399742-A	D-2392348-A	D-2407775-A	D-2411454-A	D-2394798-A	D-2410379-A	ND
	Putative rod shape-determining protein	ND	ND	V-1702207-I	ND	V-1694983-I	ND	ND
	Putative siderophore biosynthesis protein	ND	ND	ND	V-105211-I	ND	V-104967-I	ND
	Putative surface anchored protein	D-1824043-Y, E-2727039-G	T-2204398-M	K-1821786-E	K-1825420-E	K-1814294-E, MH-2722329-V	K-1834718-E	ND
	Putative thiol peroxidase	ND	ND	ND	E-1771577-K	ND	E-1770875-K	ND
	Putative transport protein	ND	ND	H-2232656-Y	ND	H-2219993-Y	ND	H-2084198-Y
	Putative transporter protein	ND	ND	ND	R-2699781-L	ND	R-2698564-L	ND

General function	Proteins encoded by genes harbouring missense mutations ¹	Amino acid transitions ²				
		2006		2011		
		R005	R016	R022	R060	R017 R030 R058
Putative transposase	A-36524,36525-G, E-36468-V, H-36922-N, H-36470,36471-R, L36548,36550-I, R-36581-S, Q, KF-37097-K, L-36548,36550-I, N-37090-D, T-37045-A	A-36675-D, A-36524,36525-G, A-36689-X, E-36468-V, H-36470,36471-R, L-36548,36550-I, R-36581-S, T-36585-I	A-36524,36525G, E-36468-V, H-36470,36471-R, L-36548,36550-I, R-36581-S, T-36585-I	ND	A-36562-D, A-36411,36412-G, A-36576,36578-S, D-36545-N, E-36355-V, H-36357,36358-R, K-36549-X, L-36435,36437-I, R-36468-S, T-36472-I	ND
SpoU rRNA Methylase family protein	ND	ND	V-1067550-I	ND	V-1064561-I	ND

¹ Missense mutations with a coverage <40 in the whole-genome sequencing analysis were excluded from the analysis.

² Amino acid transitions are indicated with the first letter indicating the amino acid encoded by the ST22-MRSA-IV reference strain M07/0319. The number indicates the nucleotide position within the whole-genome sequence of the 2006 isolate indicated and the final letter indicates the amino acid encoded by the 2006 isolate.

Abbreviations: A, Alanine; B, Aspartic acid or Asparagine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; X, unspecified or unknown; Y, Tyrosine; Z, Glutamine or Glutamic acid. (*) Insertion of a new stop codon.

³ND, None detected.

⁴Proteins highlighted in red font were shown previously to be essential to *S. aureus* growth in BHI in other studies (Forsyth *et al.*, 2002; Valentino *et al.*, 2014).

and R030 (2011) harboured the same mutations in 30/47 genes including in an exotoxin (Histidine (H) to Y amino acid transition), a nucleoside permease (V to I amino acid transition) and the glucose inhibited division protein B (Aspartic acid (D) to Asparagine (N) amino acid transition).

A single gene encoding uracil phosphoribosyltransferase exhibited the same methionine (M) to threonine (T) amino acid transition in all seven isolates. Ten genes contained missense mutations found in six isolates each and in eight of these it was the same single mutation in each of the six isolates (Table 6.5).

6.4 Discussion

Despite the ongoing predominance of ST22-MRSA-IV among *S. aureus* BSIs in Ireland the prevalence of MRSA causing BSIs declined significantly in recent years (Chapter 5). However, analysis of the virulence and antimicrobial resistance gene content of ST22-MRSA-IV isolates identified no possible causative factor for the decline in ST22-MRSA-IV (Chapter 5). The initial observation of a lack of detection of the biofilm-associated *icaC* gene from the *ica* operon in the earlier ST22-MRSA-IV isolates was due to a mutation in the DNA microarray primer binding site for *icaC*. This mutation however was identified in all of the ST22-MRSA-IV isolates from both 2006/07 and 2011 which were investigated by WGS, including those that yielded both positive and negative signals for *icaC*, as well as in other ST22-MRSA-IV isolates recovered from patients in Irish hospitals in earlier years from another study (data not shown) and does not explain the significant decrease in detection of *icaC* during both study periods. DNA microarray investigations were repeated on 30 isolates from 2006 (data not shown) which confirmed the results previously found in terms of positive and negative signals for *icaC*. This rules out the possibility that the accuracy of conditions used for DNA microarray analysis affected the detection of *icaC* by the microarray. It is possible that other mutations located outside of these genes affected *icaC* primer binding in these isolates. Although no SNVs were identified within the *ica* operon in the isolates investigated, it is important to note that the SNV analysis undertaken in the ST22 isolates only examined nonsynonymous mutations within coding regions and there may be mutations outside of these regions that are causing topological conformational changes in the DNA and thus further affecting primer binding.

If conformational changes are causing the differences in detection of *icaC* it is possible that the transcription of *icaC* may also be affected by these changes. The *ica* operon is involved in the synthesis and export of proteins which produce the polymeric N-acetyl-glucosamine polysaccharide biofilm common to clinical *S. aureus* isolates (O’Gara *et al.*, 2008). Changes in *ica* expression could hypothetically hinder biofilm formation in these isolates. However, the *ica*-dependent biofilm pathway is not the only one utilised by *S. aureus* and recent studies identified an alternative pathway which is induced in methicillin resistant isolates leading to a proteinaceous biofilm mediated by a major cell wall autolysin and the fibronectin-binding proteins (Pozzi *et al.*, 2012).

Bacterial chromosomes accumulate SNVs at varying rates but it is hypothesised that the general SNV rate in *S. aureus* is somewhere between 7.9-9.6 SNVs per genome per year (Young *et al.*, 2012; Harris *et al.*, 2010). The seven ST22-MRSA-IV isolates investigated in the present study were isolated five years apart, which would yield an expected SNV rate of between 39.5 and 48 after the five years if the 2011 isolates were directly related to those from 2006/07. The ST22-MRSA-IV isolates from both years were chosen based on differing growth rates between isolates from both study periods and differences in detection of *icaC* as determined by the DNA microarray. The number of SNVs between the isolates ranged from nine to 183 suggesting that at least some of the isolates (both within and between study periods) were very closely related. This could give rise to the lack of significant differences in amino acid changes identified between the two groups. The WGS analysis utilised a reference ST22-MRSA-IV strain, HO 5096 0412 (Holden *et al.*, 2013), as a scaffold for the assembly of the seven ST22-MRSA-IV query strains. Isolate HO 5096 0412 is a representative strain of EMRSA15 (ST22-MRSA-IV) from the UK and may be slightly different to the predominant Irish strains. This may have led to some discrepancies in the sequence data from the seven Irish ST22-MRSA-IV isolates and the overall SNV rates.

Significant differences in growth rates were identified in the early exponential stages of growth between isolates from 2006/07 and 2011. Whole-genome sequencing analysis was used to compare the fastest growing isolates in 2006 to the slowest growing in 2011 to identify any SNVs which could be involved in the slower growth rate of the more recent isolates. Factors affecting growth rate in *S. aureus* are multifaceted and highly dependent on environmental conditions including temperature and growth media. Several studies have investigated the genes involved in *S. aureus* growth rates (Valentino *et al.*, 2014; Benton *et al.*, 2004; Forsyth *et al.*, 2002) and they identified over 400 essential genes for *S. aureus* growth. Of the 400 genes SNVs in 15 of these were identified in the present study (Tables 6.3 - 6.5). In those that were unique to 2006 and 2011 isolates however SNVs were identified in only one isolate in each case and it is unlikely that these are the cause of the slower growth rates detected. One gene encoding the extracellular matrix binding protein Ebh harboured mutations in three of the four 2006 isolates and no SNVs were identified in this gene among the 2011 isolates. Ebh is a large ~1.1 MDa surface protein in *S. aureus* involved in the binding of *S. aureus* to fibronectin (Kuroda *et al.*, 2001). It is growth phase regulated and under the expression of *agr*

(Clarke *et al.*, 2002). The majority of global ST22-MRSA-IV isolates investigated have been found to harbour a nonsense mutation in this gene that impacts its expression (Holden *et al.*, 2014). The *Ebh* gene found in the three of the four 2006 isolates in this study harboured SNVs which were different to that reported previously within *Ebh* of ST22-MRSA-IV isolates. The impact of these SNVs on the functioning of *Ebh* remains to be elucidated however a previous study by Clarke *et al.* (2002) found that the previously identified mutations in *Ebh* had no effect on the growth rate or pathogenicity of *S. aureus* in a murine infection model.

A large study of over 20,000 patients from nine regions in five countries from three continents found the overall incidence of *S. aureus* BSIs were not increasing and that any differences identified between countries were caused by changes in MSSA BSI rates and not MRSA (Laupland *et al.*, 2012). Thus the decline in BSIs in Ireland may not be due to a decline in the ST22-MRSA-IV but rather an increased fitness of MSSA clones. Future work could include growth rate analysis of MSSA from different time periods to investigate if there is a significant difference in the rate that the more recent MSSA are growing. Growth rates in this study were investigated in Brain Heart Infusion broth and the analysis of MRSA and MSSA growth rate in media enhanced with human blood and under other conditions would be useful to identify if the varying rates of growth identified were also to be found under other environmental conditions.

Different geographic regions are affected by genetically distinct clones with potentially differential fitness (Grundman *et al.*, 2006). The introduction of new fitter clones into an area can replace existing dominant clones. This does not appear to be the case in the Irish situation as between both study periods no increased prevalence of any other clone has been identified, in fact among BSIs the prevalence of ST22-MRSA-IV increased despite the declining overall numbers.

This investigation also only looked at changes at the genetic level, expression levels of potent virulence determinants within ST22-MRSA-IV could be investigated to see if a cause for the decline of this clone can be identified. Co-competing, co-culturing and biofilm experiments could be undertaken to investigate declining fitness of ST22-MRSA-IV isolates from earlier years when numbers of isolates were higher to isolates from more recent years when numbers had declined. It is possible that the decline in MRSA is not caused by the decline of the ST22 clone but that increased fitness among

MSSA clones could be causing them to outcompete the MRSA. Competition assays between MRSA and MSSA isolates could help to investigate if increasingly fit MSSA are the causative factor behind declining MRSA rates among BSIs in Ireland.

Chapter 7

General Discussion

7.1. Increasing prevalence and genetic diversity of *pvl*-positive *S. aureus* in Ireland

The results of the present study highlight the increasing prevalence and diversity of *pvl*-positive *S. aureus* in Ireland along with temporal shifts in the predominant clones between 2002 and 2012. This study has provided clear evidence of distinct populations of *pvl*-positive *S. aureus* clones existing within the community in Ireland compared to hospitals, but also of the spread of *pvl*-positive *S. aureus* strains into hospitals e.g. the ST772-MRSA-V NICU cluster. However, since all CA-*S. aureus* do not carry *pvl* the burden of *S. aureus* that exists in the community in Ireland is probably still underestimated and studies are required to investigate this further particularly among high-risk groups (Rossney *et al.*, 2007; Tong *et al.*, 2009). The recent identification of a *pvl*-negative MRSA strain with a genetic background common among CA-MRSA further highlights this issue. This CC1-MRSA-IV strain has been responsible for a protracted and ongoing outbreak in one large acute Dublin hospital and has also spread to other hospitals (personal communication, Grainne Brennan, NMRSARL). The threat posed by this CA-MRSA clone in Irish hospitals is further complicated by its multidrug resistance, which includes high-level mupirocin resistance, making decolonisation very difficult. This CA-MRSA clone has the potential to displace the currently predominant nosocomial clone, ST22-MRSA-IV. In many regions, including Ireland, there are currently no national data on the burden of CA-MRSA. Several studies in other countries have identified high risk groups for CA-*S. aureus* infections including various ethnic groups e.g. African pygmies, Aborigines and Native Americans as well as prisoners in jails, children in creches, intravenous drug users and members of sports teams (Tong *et al.*, 2009; Ruimy *et al.*, 2010; Schaumburg *et al.*, 2011). Further studies should include an investigation and identification of high-risk groups for CA-*S. aureus* infections in Ireland so that infection control measures can be implemented to prevent further spread of CA-*S. aureus* in hospital and communities.

The increase in multidrug resistance (MDR) identified among the *pvl*-positive MRSA isolates in the present study is also a cause for concern and is in line with recent studies from other regions (Ellington *et al.*, 2010). This combination of multidrug resistance and increased virulence in CA-MRSA poses a serious threat to public health, not just in the community but also among already ill hospitalised patients. The increased

virulence of CA-*S. aureus* is apparent from the younger age of the patients from whom the *pvl*-positive isolates were recovered from in the present study with 67% aged less than 40 years. In comparison HCA-*S. aureus* tend to cause infections in already vulnerable patients, particularly the very old and the very young. The increase in MDR among *pvl*-positive MRSA may be the result of an increase in antibiotic usage in the community in Ireland (Health Protection Surveillance Centre (HPSC), 2013). In 2013, Ireland had the highest rate of antimicrobial consumption in the community in the EU (23.8 defined daily doses per 1000 inhabitants per day) and this was also the highest level recorded to date for Ireland (HPSC, 2013). According to the HPSC report penicillins accounted for the largest group of antimicrobials used (55.1% of total), followed by macrolides (18.5%), tetracyclines (12.5%), cephalosporins (5.7%) and fluoroquinolones (3.7%) (HPSC, 2013). Interestingly, this correlates with the high rates of resistance to macrolides and tetracycline detected among the *pvl*-positive MRSA isolates in the present study (Fig. 3.5). However, the high levels of ciprofloxacin resistance detected among the *pvl*-positive MRSA isolates is not reflected in this community antimicrobial consumption data but may reflect earlier community antimicrobial consumption patterns. Alternatively other agents may be inducing this increase in ciprofloxacin resistance. For example, triclosan, a biocide found in soaps, toothpaste, household cleaners and medical equipment, has been shown to induce increased resistance to a range of antimicrobial agents in *S. aureus* (Halden & Paull, 2005; Seaman *et al.*, 2007). The increase in ciprofloxacin resistance among *pvl*-positive MRSA also highlights the fact that, similar to the presence or absence of *pvl*, ciprofloxacin susceptibility cannot be used as a marker for CA-MRSA and that definitive identification of isolates requires both clinical and molecular epidemiological data. Increasing antimicrobial agent consumption may be driving the increase in multiresistant *pvl*-positive isolates or it may also be reflective of the greater need for antimicrobials due to these increasingly multiresistant isolates. Further surveillance is required to monitor this increasing trend of MDR MRSA in the community and to prevent the spread of these highly virulent and resistant strains in hospitals.

This study has also highlighted the need for ongoing surveillance of CA-MSSA isolates. Although the *pvl*-positive MSSA isolates investigated harboured less antimicrobial resistance genes than the MRSA and fewer MSSA isolates were investigated, they were responsible for over half of the more serious manifestations of disease (Fig. 3.4), a phenomenon that is being increasingly reported (Catena *et al.*, 2012).

While this may reflect an increased pathogenicity of *pvl*-positive MSSA it may also be that only MSSA isolates from serious manifestations were submitted to the NMRSARL for investigation. The burden of CA-MSSA in the community is likely to be much greater than identified in the present study. Several countries have identified a higher prevalence of *pvl*-positive MSSA than of *pvl*-positive MRSA, including Russia, Cameroon, Madagascar, Niger and Senegal (Baranovich *et al.*, 2009; Breurec *et al.*, 2010). It is evident that future studies of community and *pvl*-positive *S. aureus* prevalence should not focus solely on MRSA.

With the increasing prevalence of MDR and highly virulent CA-*S. aureus* it is more important than ever that healthcare workers (HCWs) are considered in the nosocomial transmission of *S. aureus*, particularly with the spread of CA-*S. aureus* in hospitals. The identification of a HCW as the index case for an outbreak of a MDR *pvl*-positive MRSA in a NICU in the present study further highlights this. Routine screening of HCWs in Irish hospitals is not performed and current Irish guidelines recommend that HCWs be screened for MRSA carriage only as part of an outbreak investigation (MRSA Guideline Committee, 2013). Similar to the practice in the Netherlands of screening all patients who have previously been hospitalised in foreign countries and all healthcare staff, identifying and screening high-risk HCWs with previous hospitalisation or a MRSA-positive family contact should be considered in Ireland (Voss *et al.*, 2005).

7.1.2 Importation of *pvl*-positive *S. aureus* clones into Ireland

The present study has also revealed that the local emergence of *pvl*-positive *S. aureus* clones in Ireland is relatively rare but that the importation of clones is common with links detected between many patients and travel to, or ethnic origin in, specific regions of the world where these clones predominate (Table 3.4). Direct correlations between travel and the introduction of new strains to a region have been reported previously (Dsouza *et al.*, 2010; Ellington *et al.*, 2010). This can be clearly seen in Ireland in the case of the ST772-MRSA-V or Bengal Bay clone where many people from whom this strain was recovered had either travelled to or had familial links with India (Tables 3.4 & 3.5). Originally identified in Bangladesh, ST772-MRSA-IV predominates in both hospitals and the community in India (Alfroz *et al.*, 2008). Since its first identification in 2008, ST772-MRSA-V has been associated with infections in Germany, Malaysia, Italy, Hong Kong,

England, Abu Dhabi and Australia (David *et al.*, 2006; Alfroz *et al.*, 2008; Neela *et al.*, 2009; D'Souza *et al.*, 2010; Ellington *et al.*, 2010; Monecke *et al.*, 2011).

The most predominant *pvl*-positive clone identified in this study, ST8-MRSA-IV, was in many instances associated with patients with a history of travel to USA, Africa, Asia, New Zealand and South America, reflecting the pandemic nature of this clone (Table 3.4). The South-West Pacific clone (CC/ST30-MRSA-IV) was the second most common *pvl*-positive MRSA clone identified and interestingly in the present study a link between travel to, or ethnic origin in, Asia or Africa was identified for several CC/ST30-MRSA-IV isolates. Isolates of these clones were also associated with familial outbreaks, indicating their propensity to spread (Table 3.4).

This is not the first time that the importation of MRSA has been reported in Ireland. As early as 1985 the introduction and spread of MRSA which were very similar to MRSA found in Baghdad was identified and subsequently found to be ST239-MRSA-III (Carroll *et al.*, 1989; Shore *et al.*, 2005). Within 18 months it had infected 65 patients within a Dublin hospital and subsequently spread to a second hospital. Similarly in 2004 ST239-MRSA-III was again imported from Singapore via a patient with a history of hospitalisation in Singapore and the strain subsequently spread to four other patients within an Irish hospital (Humphreys *et al.*, 2004; Shore *et al.*, 2005). Foreign travel as well as the employment of healthcare staff from foreign countries may lead to further importation incidents of MRSA strains into Ireland. A full cost benefit analysis is required to determine if screening of HCWs, either routinely or specifically for those with risk factors for MRSA carriage would be economically feasible.

Immigration has long been associated with shaping the epidemiology of infectious diseases globally (Gushulak & MacPherson, 2004). The extensive genetic diversity observed among *pvl*-positive *S. aureus* in Ireland is likely to be caused by a multiplicity of social and cultural behaviours and habits, technology, transport and geographic location. Rolo *et al.* (2012) hypothesised that the frequency and destination of travel, infection prevention and control measures, antibiotic prescription and consumption policies and hygiene habits all play a role in shaping the population structure of CA-MRSA in a country. The prevention of MRSA transmission in both hospitals and the community is essential for patient safety and a reduction in the costs associated with MRSA infection. Recent European studies indicated the average increased cost of each

MRSA-patient within the health care sector to be in the range of €5,700 to €10,000 (Wernitz *et al.*, 2005; Kanerva *et al.*, 2007). Early detection is essential to inform effective strategies for the control and prevention of MRSA and screening plays a pivotal role in this. Routine universal screening is not undertaken for patients on admission to hospitals in Ireland. Scandinavian countries and the Netherlands, countries with some of the lowest MRSA rates in Europe, employ a search and destroy policy to combat MRSA spread (van Rijen & Kluytmans, 2009). This involves the screening and isolation of all patients at risk of MRSA carriage including patients with a history of hospitalisation in a foreign country and those with exposure to certain livestock (Wertheim *et al.*, 2008). With the current influx of migrants into Europe from North Africa, Syria and Afghanistan, it is inevitable that Europe and Ireland will face further importation of new strains. As is evident from ST772-MRSA-V, these newly introduced clones have the potential to spread widely and cause invasive infections. Screening policies are, now more than ever, essential to identify and respond to new waves of *S. aureus* clones. These “search and destroy” policies have been found to be cost effective in countries with low MRSA incidence (van Rijen & Kluytmans, 2009). However, the cost associated with the implementation and the facilities required, such as availability of isolation rooms, may prohibit their use in countries such as Ireland where MRSA is endemic. Enforcing screening policies comes with inherent difficulties, the screening of health care staff is a controversial issue where staff do not want to be associated with the stigma of being a risk to patients (Albrich & Harbarth, 2008). The alternative to this was highlighted in the present study where an Indian healthcare worker transmitted ST772-MRSA-IV to neonates in an ICU. Systemic and compulsory screening of migrants and visa applicants is undertaken in many countries globally (CDC 2011; Australian Department of Immigration, 2014). Whilst this can be essential to ensure migrants do not import new *S. aureus* strains to the host population some countries enforce exclusion policies based on infectious disease status. Despite this medical screening, whether for healthcare staff or migrants, if done with the appropriate sensitivity, anonymity and human rights safeguards, can contribute to the health needs of patients and staff, migrants and host communities.

7.2 Diversity of *pvl*-encoding phage among *pvl*-positive MRSA

Pvl phage, with diverse genetic organisation, were identified among ST8-MRSA-IV (USA300) and ST30-MRSA-IV (Figure 4.1) isolates in the present study while a unique *pvl*-phage was detected among ST772-MRSA-V isolates (Bengal Bay clone). The range of *pvl* phage identified within ST8-MRSA-IV and ST30-MRSA-IV which were among the most commonly identified *pvl*-positive MRSA clones identified in Ireland, reflects the diversity present within this population as these are two of the most widespread pandemic CA-MRSA clones and provides further evidence that multiple importations of these clones into Ireland has occurred. There was no evidence of local transmission of *pvl* phage identified in the present study, ie a unique phage variant was not identified within different clonal backgrounds, however numbers of isolates investigated were low and future studies may highlight more changes.

It has been hypothesised previously that the unique ST772 phage arose from a single recombination event where the structural capsid and tail genes and *sea* from a *hly*-converting phage formed a hybrid with a *pvl*-encoding phage in ST772-MRSA-V (Prabhakara *et al.*, 2013). The three *pvl* phage identified among the ST772-MRSA-V isolates investigated in the present study were identical in their coding regions reflecting the recent emergence of this strain and the lack of diversity among the ST772-MRSA-V isolates. The presence of genes encoding a potent superantigen and a cytolytic toxin (*pvl*) on a single phage raises concerns of the possible spread of this phage into other very successful pandemic MRSA clones. It also highlights the potential for further recombination events between *pvl*-encoding phage and other phage-encoded virulence factors, such as the enterotoxins, leukocidins and IEC genes, many of which have been identified among isolates in the present study. Ongoing surveillance of MGEs and phage harboured by predominant *S. aureus* clones in Ireland and perhaps internationally is essential to detect any novel recombination events which could give rise to hybrid phages that could spread widely among other MRSA clones increasing their virulence potential.

7.3 Increasing diversity and virulence of MSSA and presistence of ST22-MRSA-IV among BSIs in Ireland between 2006/07 and 2011

The present study revealed that ST22-MRSA-IV continues to predominate among MRSA from BSIs while MSSA are more genetically diverse (Table 5.3). It has been suggested that this is because MSSA are much older on an evolutionary time scale than MRSA and thus are more diverse (Grundmann *et al.*, 2010). However, an increase in the diversity of MSSA from BSIs between 2006 and 2011 was identified i.e. 54 MSSA *spa* types in 2006 compared to 74 in 2011. There were also more *spa* types unique to 2011 than to 2006 and it is possible that this increasing diversity among MSSA provides them with an advantage and allowing them to outcompete MRSA.

The patient profile of those from whom MRSA and MSSA were isolated also differed between both study periods (Table 5.5). There was an increase in the number of males from which isolates were recovered in 2011 compared to 2006/07 for both MRSA and MSSA. In 2011 there was an increase in community onset MSSA infections with 52.3% of patients with MSSA BSIs in 2011 presenting from the community. Patients with invasive MRSA infections were older than those with MSSA infection and also MRSA infections had a higher mortality after 14 days than MSSA (Table 5.5). This may be due to the increased morbidity and decreased health status associated with an older patient cohort. The MSSA isolates seem to be affecting a younger, healthier more mobile cohort of patients where they have more opportunities to spread widely, while the MRSA are infecting an older, more limited and less mobile patient cohort.

Interestingly, the MSSA isolates harboured less resistance genes than the MRSA isolates but harboured more virulence genes (Tables 5.3 & 5.4) and this has also been identified in other studies (Miller *et al.*, 2007; Collins *et al.*, 2010; Jimenez *et al.*, 2011). There were differences in MSSA virulence gene carriage between the two study periods, 2006 and 2011, with a significant decrease in the carriage of the enterotoxin gene cluster in 2011 and an increase in the carriage of the potent superantigen-encoding *sea* (Fig 5.3 (b)). The increased genetic diversity and changes in virulence gene carriage among MSSA isolates may be providing a fitness advantage over the MRSA isolates and further work including competition assays, are required to investigate this further. MSSA

isolates are not routinely submitted to the NMRSARL and this potential reservoir of infection within the community should also be routinely monitored.

7.3.1 Possible evidence of ST22-MRSA-IV clonal decline

In Ireland over the last eight years there has been a 63% reduction in the numbers of reported MRSA isolates from BSIs in Ireland, from 592 in 2006 to 218 in 2014 (HSE and HPSC Report, 2015), but ST22-MRSA-IV remains the predominant strain and consistently accounts for approximately 80% of MRSA isolates from BSIs. Despite investigating an extensive range of virulence and resistance genes, the only significant difference identified between ST22-MRSA-IV isolates recovered in 2006 and 2009/10 and 2011 was the reduction in the detection of *icaC* (Fig 5.3 (a) & Fig 6.3). The *icaADBC* genes are involved in the synthesis and export of polymeric N-acetyl-glucosamine during biofilm formation by MRSA. The ability to form a biofilm is a key pathogenic feature of MRSA and it can do this by several mechanisms, including via *ica*-independent biofilm formation (O’Gara, 2007). Further investigations of the sequence of *icaC* among a selection of ST22-MRSA-IV investigated by WGS identified a single base difference in the microarray primer binding site of *icaC*, which may have affected the detection of *icaC* among these isolates. However, the prevalence of the allelic variant was identified in all seven ST22-MRSA-IV isolates that underwent WGS regardless of a positive or negative DNA microarray result for *icaC*. Repeated DNA microarray experiments confirmed the positive/negative *icaC* results in all isolates. It is possible that additional SNPs upstream or downstream of *icaC*, and not detected using our SNP analysis method which investigated only nonsynonymous SNPs in coding regions, are affecting *icaC* primer binding within these isolates. Further work is needed to elucidate whether the presence of this allelic variant results in a phenotypic difference in the ability of these strains to produce a biofilm. However, as MRSA have been found to utilise an alternative biofilm formation pathway (Pozzi *et al.*, 2012) it is unlikely that this change affects the ability of ST22-MRSA-IV isolates to cause invasive infection.

The growth rate of a selection of ST22-MRSA-IV isolates identified significant declines in the growth rate during the early stages of growth among the more recent 2011 isolates when compared to isolates from 2009 or 2006 (Fig 6.5). It was speculated that the mutation in *icaC* may be indicative of further genome wide SNPs within the most recent ST22-MRSA-IV isolates which could contribute to the slower growth rate and overall

clonal decline. Whole-genome sequencing of seven ST22-MRSA-IV isolates, four recovered from patients in 2006 and three from patients in 2011, identified a total of 348 missense mutations in 47 genes (Tables 6.3, 6.4 & 6.5). The genes were relating to a variety of essential functions in *S. aureus* including biofilm, adhesion, antimicrobial resistance, structural components, metabolism and virulence. Fifteen of the genes had previously been identified as essential for *S. aureus* growth in BHI medium (Forsyth *et al.*, 2002; Valentino *et al.*, 2014). Despite this no overall factor was identified which could be responsible for the slower growth rate of the more recent ST22-MRSA-IV isolates. The reason for the decline of ST22-MRSA-IV appears to be multifaceted with multiple gene acquisition events and mutations as opposed to individual determinants being responsible. Variations in gene expression and regulation, which were not investigated here, may also play a role in the clonal decline of ST22-MRSA-IV. The limited numbers of isolates used for the WGS leave open the possibility that a general trend within a gene, or genes, has not yet been identified. Future studies should include greater numbers of isolates and examine SNVs in non-coding regions which may have the potential to affect gene expression. An average SNV difference of 82.5 among coding regions between all seven isolates highlights the highly clonal nature of the ST22-MRSA-IV population in Irish hospitals.

7.4. The potential of whole-genome sequencing for differentiating and tracking MRSA

In the present study *spa* typing and DNA microarray profiling proved very useful for genotypic characterisation of MRSA and MSSA. Whole-genome sequencing was used for the characterisation of the genetic organisation of a MGE i.e. the *pvl*-encoding bacteriophage and for SNV comparison of ST22-MRSA-IV isolates from different study periods. In recent years advances in WGS technology have resulted in its application for typing and tracking of *S. aureus* in several studies. For example, Harris *et al.*, 2012, investigated an outbreak of MRSA in a special care baby unit in the UK. Using WGS they were able to show that transmission had occurred during the outbreak between mothers on the postnatal ward and among their families and contacts in the community. They identified a novel MRSA ST2371, related to ST22 which had caused the outbreak. Similar to the present study they identified a staff member who carried MRSA and facilitated the transmission of the strain even after thorough environmental cleaning and infection prevention and control measures had been implemented. Whole-genome

sequencing, and SNV analysis, has huge potential to improve tracking and typing of *S. aureus*, adding increased discrimination potential and allowing for detection of the original 'patient zero' or ongoing source of infection. The evolutionary rate of *S. aureus* is estimated to be in the order of 10^{-6} substitutions/site/year (Harris *et al.*, 2010; Nubel *et al.*, 2010) though this has been debated and may be clone and site specific. Different studies use different threshold limits to define isolate relatedness (Harris *et al.*, 2010; Nubel *et al.*, 2010) and further work is required to determine the mutation rates of individual clones before WGS can be applied routinely. Despite the clear benefits, the associated cost and computational resources required still remain prohibitive to routine use of these methods.

7.5 Concluding remarks

Overall this study has highlighted key changes in the molecular epidemiology of *S. aureus* infections within both the healthcare and community settings in Ireland. Further work is required to determine if the MSSA have the capacity to outcompete the dominant HCA-MRSA. Only MRSA from BSIs were investigated; MRSA cause a multitude of invasive infections and perhaps the declining trends in MRSA numbers are not reflected in these manifestations. The results of this study highlight the need for ongoing surveillance of both MRSA and MSSA in hospitals and communities in Ireland, particularly with the recent increase in prevalence and diversity of MSSA and *pvl*-positive *S. aureus* in the community setting. There was no evidence of MRSA strain replacement among BSIs, however this has happened on numerous occasions in the past and is likely to happen again with the increased importation of new clones. WGS will revolutionise clinical diagnostics and will in the future provide unprecedented insights into the spread of clones.

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