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Antibiotic resistance, virulence and clonal diversity of methicillin resistant and sensitive *Staphylococcus aureus* isolates

PhD thesis

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LIST OF ABBREVIATIONS

ACME - Arginine-catabolic mobile element

AME - Aminoglycoside-modifying enzyme

AUC - Area under the curve

BSI – Bloodstream infection

CA-MRSA – Community- associated methicillin resistant *S. aureus*

CC - Clonal complex

CCI - Charlson comorbidity index

HA-MRSA – Hospital- associated methicillin resistant *S. aureus*

hVISA - Heterogeneous VISA

LA-MRSA – Livestock-associated methicillin resistant *S. aureus*

MALDI-TOF MS - Matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry

MATE - Multidrug and toxin extrusion

MDR - Multidrug resistance

MGE – Mobile genetic element

MIC - Minimum inhibitory concentration

MLS_BK - Macrolides, lincosamides, $streptogramin_B$ and ketolids

MLST - Multilocus sequence typing

MRSA - Methicillin resistant *S. aureus*

MSSA - Methicillin sensitive *S. aureus*

MSCRAMM - Microbial surface components recognizing adhesive matrix molecules

OD - Optical density

PBP - Penicillin binding protein

PCR - Polymerase chain reaction

PFGE - Pulsed-field gel electrophoresis

PVL – Panton-Valentin leucocidin

QRDR - Quinolone resistance-determining region

SAB- *Staphylococcus aureus* bacteraemia

SaPI – *Staphylococcus aureus* pathogenicity island

SCC*mec* - Staphylococcal cassette chromosome *mec*

Spa – Staphylococcus protein A

SSTI - Superficial skin and soft tissue infections

SSSS - Staphylococcal scalded skin syndrome

ST – Sequence type

TSS - Toxic shock syndrome

VISA - Vancomycin intermediate-resistant *S. aureus*

VRSA - Vancomycin resistant *S. aureus*

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1. INTRODUCTION

1.1. Background

Staphylococcus aureus is one of the most important human pathogens. It is widespread all over the world, often colonising the anterior nares, skin, oropharynx, intestinal tract and vagina of healthy individuals (1). It causes various pyogenic and toxin mediated diseases with high mortality. Most important diseases caused by *S. aureus* include skin and soft tissue infections, osteoarticular infections, abscesses, prostatic device infections, infective endocarditis, food poisoning, bacteraemia and sepsis (2).

S. aureus is a Gram-positive coccus of 1 µm, generally arranged in grape-like cluster due to replication in random division planes. It belongs to the family of *Staphylococcaceae*, and is part of the *Staphylococcus* genus, which contains 54 species and 28 subspecies (3). *S. aureus* is the most virulent species of the family and is part of the *S. aureus*-complex with *S. argenteus* and *S. sweitzeri*. These novel species are nearly identical in their 16S rRNA gene sequences and are the closest relatives of *S. aureus*, however, they are classified into different species based on their phylogenetic distance and other differences (4) (Figure 1).

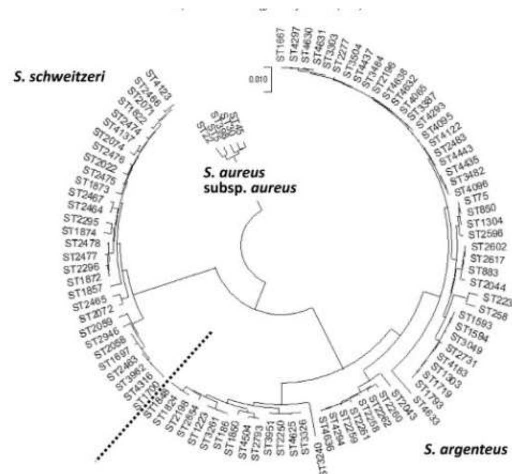

Figure 1. Phylogenetic tree of the *S. aureus*-complex (5)

S. aureus is one of the first described human pathogens. It was first isolated by Alexander Ogston from a surgical wound infection in 1880. The isolated organism was able to cause

purulent inflammation when injected into guinea pigs and mice, an experiment later repeated by Louis Pasteur (6). In 1884 Rosenbach divided the *Staphylococcus* genus into *S. aureus* and *S. albus*, which was later named *Staphylococcus epidermidis* (7).

1.2. Epidemiology of *S. aureus* infections

Up to 30% of asymptomatic, healthy individuals are colonised by *S. aureus*, mainly in the anterior nares (8). Asymptomatic carriers have an increased risk for subsequent infection (9). Colonisation rates change throughout the life and peak around the age of 10 years (10). Asymptomatic carriers are an important source of human-to-human transmission of the bacterium, especially in healthcare settings. Fomites, such as pens and coats also play an important role in the hospital spread of the pathogen (11). *S. aureus* is also frequently present in home environment, in a study 50% of the tested bedrooms were contaminated with the bacterium (12).

For *S. aureus* bacteraemia (SAB), the best-described manifestation of the infection, population incidence ranges from 10 to 30 per 100,000 person-years (2). Incidence increased from the 1950s to 1990 and stabilised around 20 per 100,000 person-years since then (2). In the recent years, growing number of healthcare-associated infections, particularly endocarditis and device related infections were reported. Community acquired skin and soft tissue infections also increase in prevalence (2).

Case fatality rate (CFR) for SAB remained between 15-50% in the past decades. Lack of improvement in survival rate is the results of the increase of older, more immunosuppressed population, and also the result of lacking new antibiotic options and increasing antibiotic resistance (2).

1.3. Major virulence factors of *S. aureus*

S. aureus was and remains one of the most common causes of infections in humans (13). It is well known for its capacity to produce an armada of virulence factors to overcome host defence systems and cause diseases. Among the many virulence factors, *S. aureus* toxins, enzymes and immune-evasive surface factors are the most important types (Table 1).

Many of the virulence factors are encoded on the accessory genome of the bacterium, which is much more variable compared to the core genome. Accessory genome consists of mobile genetic elements (MGEs) such as *S. aureus* pathogenicity islands (SaPIs), bacteriophages, chromosomal cassettes, transposons and plasmids, which are transmitted by horizontal transfer between strains (11).

1.3.1. Cell surface associated adhesins

S. aureus expresses many cell-surface associated adhesins termed '**microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs)**, allowing the bacterium to bind to extracellular matrix proteins (ECM) of the host, contributing to invasion and infection. **Polysaccharide intercellular adhesin (PIA)**, also referred as poly N-acetylglucosamine (PNAG) mediates bacterial adhesion and is important component of the staphylococcal biofilm. PIA is synthetized by N-acetylglucosamyl transferase, the product of *icaA* gene (14). **Collagen binding protein (CNA)** has an important role in the pathogenesis of *S. aureus*, enhancing the adherence of the bacterium to connective tissue, and thus cause wound, skin and soft tissue infections (15). CNA also prevents activation of complement system through the classical pathway (16). **Staphylococcus Protein A (SpA)** is produced by the vast majority of clinical *S. aureus* strains and supresses immune response by binding to Fc and Fab domains of IgG antibodies (17).

1.3.2. Staphylococcus aureus toxins

Pore-forming toxins (PFTs), including hemolysin-α, β, and ɣ, and leukotoxins such as Panton-Valentine leucocidin are successful in lysing a variety of human cells, among others epithelial cells, endothelial cells, T cells, monocytes and macrophages (18). Therefore, they support the bacterium to cause and maintain the infection, enhance the spread in the tissue and avoid immune response.

Hemolysin-α (Hla) is among the most frequently secreted staphylococcal toxins. It stimulates the lysis of a wide range of human cells and disrupts the integrity of epithelial and endothelial layers, enhancing invasion of host tissue by the bacterium. Hla intensely

contributes to the pathogenesis of skin infections and pneumonia (18-20). ***Hemolysin β (Hlb)*** is cytotoxic to leukocytes, lymphocytes, monocytes and keratinocytes. It inhibits interleukin-8 (IL-8) expression, contributes to phagosomal escape and biofilm formation of *S. aureus* (18). ***Hemolysin γ (Hlg)*** is a group of proteins with different subunit combinations. Through their lytic effect, these toxins help the bacterium to evade macrophages and release iron, an important nutrient from erythrocytes, thus they have strong role in *S. aureus* survival and replication in bloodstream infections (18, 21). ***Panton-Valentine leukocidin (PVL)*** is a two-component cytotoxin associated with severe necrotising pneumonia, osteomyelitis, skin and soft tissue infections, produced by approximately 5% of *S. aureus* strains (22-24). ***Exfoliative toxin A and*** **B (ETA, ETB)** are specific serine proteases cleaving desmosomes in keratinocyte junctions leading to staphylococcal scalded skin syndrome (25). ***Superantigens (SAgs)*** of *S. aureus* hyperactivate T-lymphocytes causing cytokine storm leading to manifestation of severe systemic symptoms as high fever, rash, hypotension etc. The nomenclature of SAgs established in 2004 distinguishes **toxic shock syndrome toxin (TSST-1)** from **staphylococcal enterotoxins (SEs)** capable of eliciting vomiting and diarrhoea after oral uptake, and from staphylococcal enterotoxin-like toxins (SEls) (19, 26).

Table 1. Most common virulence factors of *S. aureus (11, 16, 27)*

Gene name	Gene product	Function	Location
MSCRAMMs: microbial surface components recognizing adhesive matrix molecules			
spa	*Staphylococcus* protein A (Spa)	Binds to immunoglobulins - Immune evasion	Core genom
cna	Collagen adhesin (Cna)	Adhesin binding to collagen	Core genom
icaA	N-acetylglucoseamino-transferase enzyme	Synthesis of polysaccharide intercellular adhesin (PIA) and capsular polysaccharide/adhesin (PS/A)	Core genom
ebhA, B	Extracellular matrix-binding proteins	Adhesion	Core genom
clfA, B	Fibrinogen binding proteins	Adhesion	Core genom

Superantigens			
sea	Enterotoxin A	Enterotoxin, superantigen	Bacteriophage
seb	Enterotoxin B	Enterotoxin, superantigen	Pathogenicity island
sec	Enterotoxin C	Enterotoxin, superantigen	Pathogenicity island
seq2,sek2	Enterotoxin and superantigen	Enterotoxin, superantigen	Pathogenicity island
sep	Enterotoxin P	Enterotoxin, superantigen	Bacteriophage
tst	Toxic shock syndrome toxin 1 (TSST-1)	Exotoxin, superantigen	Pathogenicity island
Exfoliative toxins			
eta	Exfoliative toxin A	Exotoxin with serine protease and superantigen activity	Bacteriophage
etb	Exfoliative toxin B	Exotoxin with serine protease and superantigen activity	Plasmid
etd	Exfoliative toxin D		Genomic island
Cytotoxins			
hla	Alpha-haemolysin (Hla)	Haemolytic toxin	Core genome
hlb	Beta-haemolysin (Hlb)	Haemolytic toxin	Core genome
hld	Delta-haemolysin (Hld)	Haemolytic toxin	Core genome
hlg	Gamma-haemolysin (Hlg)	Haemolytic toxin	Core genome
hlg-v	Gamma-haemolysin variant (Hlg-v)	Haemolytic toxin	Core genome
lukD and lukE	Leukotoxins	Immune evasion	Pathogenicity island
lukS-lukF PV	Pantone-Valentine leukocidin (PVL)	Bicomponent leukocidin	Bacteriophage
Enzymes			
aur	Aureolysin	Tissue destruction	Core genom
coa	Staphylocoagulase	Coagulation	Core genom
geh	Lipase	Lipid degradation	Core genom
hysA	Hyaluronidase	Tissue invasion	Genomic island
sak	Staphylokinase	Clot dissolution	Bacteriophage
sspA	Serin protease	Tissue destruction	Core genom
sspB	Cysteine protease	Tissue destruction	Core genom

Other virulence factors			
capA* and *capB	Capsular polysaccharide biosynthesis proteins	Immune evasion	Core genome
chp	Chemotaxis inhibitory protein	Immune evasion	Bacteriophage

1.4. Pathogenicity and clinical diseases associated with *S. aureus*

S. aureus infections can be a result of auto-infection, when a person colonised with the bacterium gets infected with the own carrier strain; or cross-infection, when the bacterium is introduced into the body from another source. *S. aureus* can potentially infect any tissue in the human body. Infections can be classified into (1) superficial skin and soft tissue infections (SSTIs); (2) deep seated, systemic and life-threatening infections, as endocarditis, osteomyelitis, pneumonia, meningitis, sepsis; (3) toxicoses as food poisoning, staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS). Severity of the infection is dependent on the virulence of the particular strain, inoculum size and immunity of the infected host (28).

Initial colonisation of the bacterium is mediated by teichoic acid on the cell wall, followed by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) binding to their specific sites (Figure 2a). Interaction between *S. aureus* and other bacteria colonising skin and mucosal surfaces, as *Staphylococcus lugdunensis, S. epidermidis, Corynebacterium* spp., *Propionibacterium acnes* also has a role in this process (9). *S. aureus* can form biofilms, in which bacteria are embedded in sticky extracellular matrix. This is especially common on plastic and metal surfaces, allowing the bacterium to cause catheter-associated and joint-replacement-associated infections or ventilator-associated pneumonia.

Upon entry to the body through microlesions and wounds, *S. aureus* often leads to abscess formation, mediated by coagulase, resulting in fibrin covered localised infection (Figure 2b). This helps the bacterium evade immune response. Leukocytes in the abscess are killed by cytolytic toxins, such as leukotoxins and Panton-Valentine leukocidin.

At later stages of the infection, abscesses may get disrupted, releasing their content to the surface in a form of purulent discharge, or towards the bloodstream to cause bacteraemia

and sepsis (Figure 2c). *S. aureus* can attach to endovascular surfaces, leading to endocarditis and metastatic abscesses. Toxins of the bacteria can lyse various blood cells in the circulation and contribute of systemic coagulation. Superantigens hyperactivate immune response, leading to cytokine storm, vasodilatation, hypoperfusion and septic shock (9).

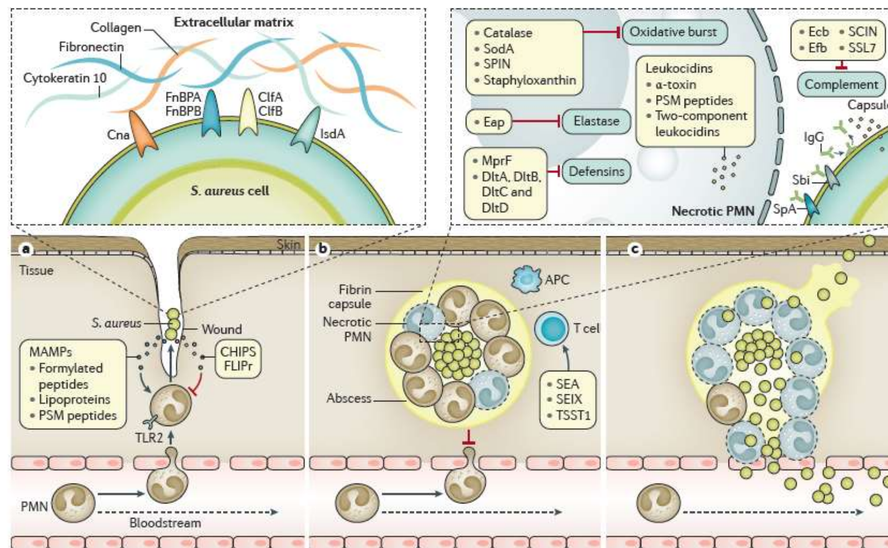

Figure 2. Stages of *S. aureus* infection (a) Initiation of the infection: entry and attachment (b) Abscess formation and evasion of immune response (c) Dissemination and systemic infection (9)

1.5. Laboratory identification of *S. aureus*

In the microbiology laboratory, *S. aureus* may be diagnosed from clinical samples from manifest infections and from screening samples. Clinical samples are collected from symptomatic patients, and include specimens of purulent discharge, tissue biopsy, sputum, blood etc. Screening samples are collected to prove or rule out asymptomatic carriage, mostly from the nasal, perianal and pharyngeal areas (9).

Basic phenotypic methods, such as microscopy and cultivation are mostly used for the laboratory identification of the bacterium. *S. aureus* is a facultative anaerobic organism, which is easy to culture. It forms golden coloured, 2-3 mm, shiny colonies and shows β-

haemolysis on blood agar. Biochemical properties of the bacterium include catalase, urease, phosphatase, lecithinase and coagulase positivity and it ferments mannitol (28).

Non-phenotypic, molecular methods are becoming increasingly important in the routine identification of bacteria. All strains of *S. aureus* produce a heat-stable thermonuclease, which can degrade DNA and RNA. This enzyme is the product of the *nucA* gene, and detection of the presence of *nucA* gene by polymerase chain reaction (PCR) identifies the bacterium as *S. aureus* (29). Matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis is based on comparison of protein profiles of bacteria. This method is also gaining importance in the laboratory identification of *S. aureus* and other pathogens (9).

1.6. Antibiotic treatment and resistance of *S. aureus*

S. aureus' success as a human pathogen is attributed to its remarkable ability to adapt to various environmental conditions. Antibiotics are not exceptions from this: *S. aureus* infections are notoriously difficult to treat due to its extensive resistance to antibiotics of various classes. *S. aureus* has the ability to become resistant to every antibiotics used in therapy (28).

1.6.1. Antibiotics acting on cell wall and cell membrane and resistance to them

1.6.1.1. β-lactams

Staphylococci have two primary resistance mechanism to overcome β-lactam antibiotics. One is expression of β-lactamase enzymes, the other one is changing the target of these antibiotics (30).

Penicillin resistant strains of *S. aureus* were discovered in 1942, soon after the introduction of the antibiotic to clinical practice (31). Resistance to penicillin can be mediated by β-lactamase enzyme, encoded by *blaZ* gene, which is located on a transposable element on a plasmid (28).

A penicillinase stable semisynthetic β-lactam, methicillin was developed in the late 1950s, however, resistance to this compound was observed within one year of clinical use

(11). Methicillin resistance is the result of a modified penicillin binding protein (PBP) with low affinity to β-lactams (PBP2a), leading to resistance to the entire class of antibiotics. This protein is encoded by the *mecA* gene in most cases, however, homologues of *mecA*: *mecB* and *mecC* genes were also described from *S. aureus* strains (32). The *mecA* gene is situated on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) (33). SCC*mec* elements carry several other components besides the *mecA* gene, such as regulatory genes, recombinases and usually genes transferring resistance to other antibiotic classes (34). MRSA has become a major human pathogen. MRSA strains typically carry multiple antibiotic resistance genes, and infections caused by MRSA strains are described to have higher mortality rates than those caused by methicillin sensitive *S. aureus* (MSSA) isolates (13).

1.6.1.2. Glycopeptides

Glycopeptides, such as vancomycin and teicoplanin are widely used in the treatment of MRSA infections. These antibiotics inhibit the cell wall synthesis by binding to the D-Ala4-D-Ala5 dipeptide, thus preventing transglycosylation and transpeptidation catalysed by PBP (35).

High level resistance in Staphylococci is luckily rare. Staphylococci can acquire the *van* genes, especially *vanA* gene from Enterococci, which encodes an enzyme replacing D-Ala5 with D-lactate in the biosynthesis of peptidoglycan, resulting in low affinity for glycopeptides (35). Vancomycin resistant *S. aureus* (VRSA) was first described in the United States in 2002, however, it remained extremely rare, most likely because of the high fitness cost associated with the acquisition of vancomycin resistance (36).

Reduced susceptibility to glycopeptides is more frequent in Staphylococci. Vancomycin intermediate-resistant *S. aureus* (VISA) emerged in 1997 in Japan, and has been reported from all over the world (37). These strains have 4-8 mg/L vancomycin minimum inhibitory concentrations (MIC), and have been associated with glycopeptide treatment failures. In VISA strains, reduced glycopeptide sensitivity is the result of increase in the thickness of the cell wall; reduction of cross-linking in the peptidoglycan; and an abundance of D-Ala-D-Ala targets sequestering the drug. VISA strains emerge during vancomycin treatment, through acquiring multiple mutations in the chromosomal genes

that affect cell wall synthesis. VISA strains develop from hVISA (heterogeneous VISA) populations, where the majority of cells are glycopeptide sensitive, however, they contain a VISA subpopulation (35).

New semisynthetic lipoglycopeptides, as dalbavancin, ortinavancin and telavancin are effective against MSSA, MRSA and VISA strains. These drugs are acting on the cell membrane and on the cell wall at the same time. Non-susceptibility to these antibiotics has also been reported (38).

1.6.1.3. Lipopeptide antibiotic

Daptomycin is a cyclic lipopeptide antibiotic, which is active against multiresistant Gram-positive bacteria, including MRSA. It disrupts cell membrane leading to permeabilisation and cell death, and also inhibits protein and nucleic acid synthesis and has immunomodulatory effects. Resistance to daptomycin is uncommon in *S. aureus*, however, it may develop during the selective pressure of antibiotic therapy. The suspected pathomechanism of resistance is changing the charge of the bacterial surface, leading to repulsion of the drug from the cell surface (39).

1.6.2. Protein synthesis inhibitor antibiotics and resistance to them

1.6.2.1. Antibiotics acting on the 30S subunit of ribosome

Tetracyclines inhibit protein synthesis by binding to the 30S subunit of the bacterial ribosome and preventing the attachment of tRNA to its recognition site. Bacteria may become resistant to tetracycline and doxycycline by producing tetA(K) or TetA(L) efflux pumps, whereas minocycline remains active in efflux pump producing strains (40, 41). Another resistance mechanism is the protection of the ribosome from the binding of the antibiotic by the elongation factor-like Tet O/M GTPase protein, which dislodges tetracyclines from the ribosome (35).

A newer glycylcyline antibiotic, tigecycline has a higher affinity for its binding site, and overcomes resistance based on ribosomal protection and efflux. There have been very few reports on tigecycline resistance in *S. aureus*. Resistance is supposed to be the result

of increased transcription of a multidrug and toxin extrusion (MATE) family transporter called MepA (40).

Aminoglycoside antibiotics have several binding sites close to the decoding centre of the ribosome and cause high rate of misreading during translation, resulting in faulty proteins and bactericidal effect (35). The most common resistance mechanism to aminoglycosides is the enzymatic modification of the drugs by aminoglycoside- phospho-transferase (APH), acetyl-transferase (AAC) or nucleotide-transferase (ANT), preventing ribosome binding (40). Aminoglycoside-modifying enzymes (AMEs) are encoded on mobile genetic elements (MGEs). Most frequent aminoglycoside resistance determinant in MRSA is the bifunctional AAC(6')-APH(2") enzyme (40).

1.6.2.2. Antibiotics acting on the 50S subunit of the ribosome

Macrolides, lincosamides, streptogramin$_B$ and ketolids (MLS$_B$K) inhibit transpeptidation and translocation on the 50S subunit of the ribosome.

Macrolides, as erythromycin and clarithromycin; and clindamycin are rarely used to treat staphylococcal infections, but may have role in therapy of MSSA infections. Most frequent type of resistance against macrolides is methylation of rRNA by Erm methyltransferases (40).

From streptogramins, quinupristin/dalfopristine combination is used in the treatment of *S. aureus* infections, and resistance rates are very low (42).

Pleuromutilins were used in veterinary medicine for decades. Recently new derivatives of the class were approved for human use. Repamutilin is a topical agent, whereas lefamulin is recommended for pneumonia and skin and soft tissue infections caused by a wide spectrum of bacteria. Resistance to pleuromutilins is very rare (43, 44).

Oxazolidinones, as linezolid and tedizolid inhibit the binding of tRNA to the ribosome. Resistance is rare, and most frequently is the result gradually acquired 23S RNA mutations (40).

1.6.2.3. Other mode of action of protein synthesis inhibition

Mupirocin is a very effective antibiotic against *S. aureus* that is available in topical formulation, its main application is the eradication of asymptomatic nasal colonisation with MRSA. It inhibits protein synthesis through binding to isoleucyl t-RNA synthetase (IleRS). Low level mupirocin resistance develops as a result of mutations in the target. High-level mupirocin resistance is transmissible by a plasmid encoding an alternative IleRS enzyme to which the drug cannot bind (35).

1.6.3. Nucleic acid synthesis inhibitors

1.6.3.1. Fluoroquinolones

Fluoroquinolones were first introduced to clinical practice in the 1990s, and had broad spectrum activity against a wide range of Gram-negative and Gram-positive bacteria (28). Resistance to fluoroquinolones quickly emerged in MRSA strains due to the frequent exposure to these antibiotics in hospital settings. The targets of fluoroquinolones in *S. aureus* are (i) the DNA gyrase, an enzyme responsible for supercoiling the chromosomal DNA and (ii) the topoisomerase IV which promotes chromosome decatenation following replication. Both consist of two subunits, GryA and GyrB in DNA gyrase and ParC and ParE in topoisomerase IV, encoded by *gyrA, gyrB* and *grlA, grlB* genes (35). Resistance against fluoroquinolones can be the result of stepwise acquisition of chromosomal mutations in the genes encoding these enzymes. Mutations in the quinolone resistance-determining region (QRDR) of ParC are especially important. Exposure to sub-inhibitory levels of fluoroquinolones allow selection of resistant mutants and induce higher mutation rates (45). *GrlA* mutations of TCC→TTC or TAC (Ser-80→Phe or Tyr) and *gyrA* mutations of TCA→TTA (Ser-84→Leu) are the principal mutations leading to fluoroquinolone resistance. Strains containing mutation in both genes become highly resistant to fluoroquinolones (46).

Another mechanism for fluoroquinolone resistance in Staphylococci is the overexpression of chromosomally encoded efflux pumps, namely NorA, NorB and NorC (35, 43).

1.6.3.2. Antibiotics inhibiting folate synthesis

Sulphonamides inhibit dihydropteroate synthetase (DHPS) and trimethoprim inhibits dihydrofolate reductase (DHFR), both being essential enzymes in the folate synthesis of prokaryotes (35). These two drugs are used in combination as co-trimoxazole in the treatment of skin and soft tissue infections caused by *S. aureus*. Resistance in Europe is rare, and mostly results from mutations in the chromosomally encoded DHFR and DHPS genes. Transmissible resistance genes *dfrK* and *dfrG* are reported from Africa and Asia (35).

Antibiotics used in the treatment of *S. aureus* infections are summarised in Table 2. Patients with mild localised MRSA infections (eg. skin and soft tissue infections) can be treated with co-trimoxazole, tetracyclines or clindamycin. For systemic infections caused by MRSA vancomycin treatment is recommended.

Table 2. Antibiotics used in the treatment of *S. aureus* infections

Antibiotics	Target	Resistance mechanism	Genes determining resistance	Examples of compounds against MSSA	Examples of compound s against MRSA
Cell wall synthesis inhibitors					
Beta-lactams	PBP	Beta-lactamase production	*blaZ*	amoxicillin+ clavulanic acid, cefuroxim	cefta-roline, cefto-biprole
		Changing the target to PBP2a	*mecA, mecB, mecC*		
Glyco-peptides	D-Ala-D-Ala dipeptide	Prevention of transglycosylation and transpeptidation (VRSA)	*vanA*	-	vanco-mycin, teico-planin
		Increased cell wall thickness and reduced cross-linking (VISA)	*mutations in graR, fdh2, sle1, rpoB, cmk etc.*		
Lipo-glycopepti des	D-Ala-D-Ala dipeptide	Increased cell wall thickness?	*gdpP?*		dalba-vancin, ortina-

					vancin, televancin
Cell membrane inhibitor					
Cyclic lipo-peptide	Cell membrane	Changing surface charge	*mprF*	-	daptomycin
Protein synthesis inhibitors					
Tetra-cyclines	30S ribosome	Efflux pump Ribosome protection by TetO/M	*tetK, tetL* *tetO, tetM*	tetracycline, doxycyline	mino-cycline, omada-cycline, erava-cycline
Glycycl-cycline	30S ribosome	Efflux pump	*mepA*		tigecycline
Amino-glycosides	30S ribosome	Aminoglycoside modifying enzymes	*aac(6')-aph(2") etc.*	gentamicin, amikacin, tobramycin	neomycin, plazomycin
Macro-lides	50S ribosome	Methylation of rRNA	*erm*	erythromycin	
Lincos-amides	50S ribosome	Methylation of rRNA	*erm*	clindamycin	
Strepto-gramin	50S ribosome	Methylation of rRNA?	*erm?*		quinu-pristin/ dalfo-pristine
Ketolids	50S ribosome	Methylation of rRNA?, efflux	*erm, mef*		telithro-mycin, solithro-mycin
Pleuro-mutilin	50S ribosome	Alternation of target	*rplC*		lefamulin, retapamuli n
Oxazoli-dinones	50S ribosome	Alternation of target	*cfr*		linezolide, tedizolide
Mupirocin	Isoleucyl t-RNA synthetase	Alternation of target Alternation of target	*ileS* *mupA*		mupirocin
Nucleic acid synthesis inhibitors					
Fluoro-quinolones	DNA gyrase	Chromosomal mutations changing the target	*gyrA, gyrB*	levofloxacin, moxifloxacin	dela-floxacin

	Topo-ismerase IV	Chromosomal mutations changing the target	*grlA, grlB*	
		Efflux pumps	*norA, NorB, norC*	
Folate synthesis inhibitors	DHPS, DHFR	Changing the target	*dfrK, dfrG*	sulphamethoxazole, trimethoprim

1.7. Clonal diversity of *S. aureus*

Population structure of MSSA strains is diverse, whereas among MRSA just a few clonal complexes predominate at a given time, at a certain geographical location, and separate clones are competing with each other for the niche.

1.7.1. Methods used for typing of *S. aureus* isolates

The epidemiology of *S. aureus* infections is changing constantly. Genetic relatedness of the isolates circulating in an environment is determined via comparison of their phenotypic and genotypic characteristics by the means of typing methods. Clonal relationship and geographical distribution of strains are followed by these methods, contributing to outbreak investigations and to the better understanding of epidemiology of the infections (13).

Historically, clonal relatedness of *S. aureus* isolates were studied by phage-typing, based on differences in the ability of bacteriophages to infect various bacterial isolates.

Pulsed-field gel electrophoresis (PFGE) was among the first genotype based methods for comparison of strains. It is based on digestion of the complete bacterial DNA genome by rare cutting restriction endonucleases, separation of the fragments by long-run gel electrophoresis and comparison of the banding patterns (pulsotypes).

Multilocus sequence typing (MLST) allows more precise and easy to compare evaluation of genetic relatedness of the strains. This method is based on the sequencing of seven housekeeping genes that are present in every strain of a given species. Based on allelic profile of these seven loci, MLST assigns a numerical sequence type (ST) to each isolate

(13). Closely related STs that match the central genotype (ST) at four or more loci are grouped into clonal complexes (CCs) (47).

In SCC*mec* typing, strains of MRSA are compared based on differences in the SCC*mec* mobile genetic element, encoding regulatory and antibiotic resistance genes. So far 13 different SCC*mec* types and several subtypes were described, labelled by roman numbers. *S. aureus* isolates belonging to the same ST or pulsotype may carry different SCC*mec* elements, and recently a consensus has been established that MRSA clones are defined by both the type of SCC*mec* element and the type of chromosome in which this element is integrated (ST), eg. ST5-II (48). SCC*mec* typing is based on multiplex PCR identification of various element of SCC*mec* cassette according to methods developed by Milheirico et al. and Zhang et al. (49, 50).

1.7.2. The most important epidemic clones of MRSA

The first MRSA strains emerged in the healthcare in the 1960s and most infections were acquired in hospitals (HA-MRSA). Later MRSA evolved in the community as well, independently from HA-MRSA (13). These strains infected individuals with no previous health-care contact, causing community-associated infection (CA-MRSA). Originally MRSA isolates harbouring SCC*mec* elements type I, II and III mostly caused hospital-acquired infections, whereas MRSA isolates with smaller SCC*mec* casettes (types IV and V) spread in the community (51). From the 2000s livestock-associated MRSA infections, acquired from animals or their products are also increasingly reported (9). However, distinctions between HA- and CA-MRSA lineages are not absolute, transfer of bacteria between these settings is increasingly recognised, as classical CA-MRSA types are becoming successful in hospital environment (52, 53).

During the molecular evolution of MRSA clones, SCC*mec* has integrated on at least 20 occasions into different lineages of MSSA (54). Majority of MRSA infections are caused by only a few successful epidemic MRSA clones. Globally, most frequently reported clones belong to five major clonal complexes, CC5, CC8, CC22, CC30 and CC45 (13) (Figure 3). Horizontal transfer of SCC*mec* between *S. aureus* isolates is very rare. Changes in the prevalence of methicillin-resistant isolates among *S. aureus* population

are the result of the changing dynamics of the spread of isolates belonging to different clones and not related to spread of methicillin resistance among *S. aureus* isolates (13).

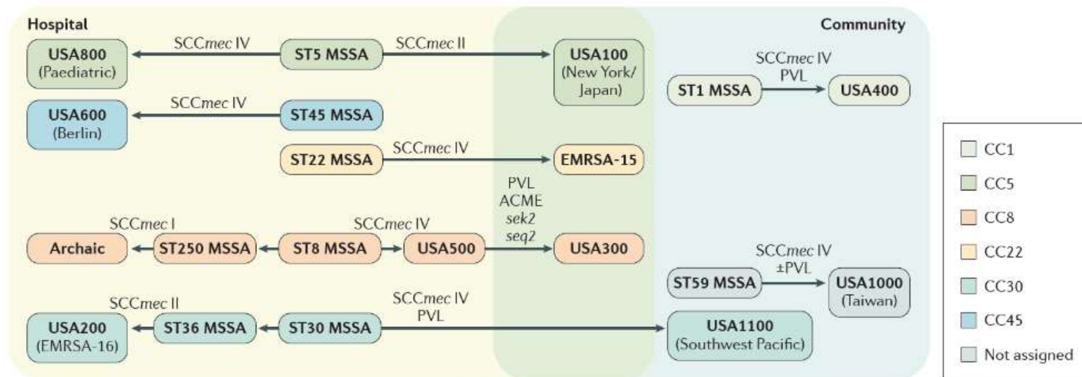

Figure 3. Origin of the most frequent MRSA clones (11)

Distribution and success of various *S. aureus* clones changes over time, and also geographically (Figure 4). In each geographical location, only one or two MRSA lineages predominate at a given time and successful clones increase in prevalence, reach their peak and then decline to be replaced by a new emerging clone (13).

The first international clone of HA-MRSA, ST5-II (New York – Japan clone) emerged and became widespread in the 1990s worldwide (55). In Hungary, during the 1990s the most frequent *S. aureus* clone was the ST239-III (Hungarian/Brazilian), later being replaced by the ST228-I (South-German) and the ST5-II (New York - Japan) clone from the beginning of the 2000s (56). Later, these clones started to decrease in prevalence and ST22-IV (EMRSA-15), an originally community-associated type started to become increasingly frequent (57). ST22-IV clone became dominant in many other parts of the world as well. According to a South-German study from 2016, this type appeared in 2001 and became rapidly common in their samples, accounting for nearly 80% of the MRSA strains by 2013 (58). It was described as the most prevalent sequence type in NICU patients in Italy (59). It replaced the Iberian and Brazilian clone in the Czech Republic (60). It has been causing nosocomial infections in the UK and in Ireland since the beginning of the 2000s (61) and was also described outside Europe, for example in Kuwait (62). However, as other successful European clones, it remained rare in the United States. On the American continent, an ST8-IV derivative, the USA300 is dominant,

whereas a related clone, USA300-LV spreads in Latin-America (11). ST398, a livestock-associated clone has been reported from Europe, Asia, Australia and the Americas since the 2000s (11).

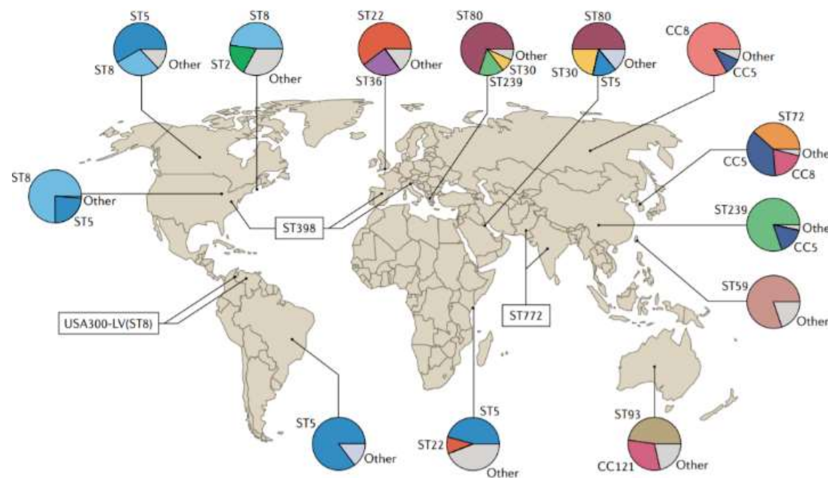

Figure 4. Clonal distribution of MRSA in the world (11)

1.7.3. Factors contributing to the changes in predominant lineages of MRSA

Despite of the great diversity of MRSA strains, relatively few clones of MRSA cause most of the infections and become successful internationally. The phenomenon of the clonal replacement, i.e. the change in the dominant clones over time, is studied extensively to identify driving forces of the population dynamics of *S. aureus*.

Earlier studies focused on the virulence determinants of bacteria. Emergence and success of new types was attributed to increased virulence due to the presence of multiple MGEs carrying virulence factor genes. Another factor hypothesized to determine the success of a given strain was the ACME (arginine-catabolic mobile element), found in the USA300 clone. Arginine deaminase enzyme inhibits innate and adaptive immune response, thus presumed to increase the ability of the bacterium to cause and maintain an infection. However, further studies found that these factors do not entirely explain the success of USA300 strains (11).

Acquisition of antibiotic resistance is a necessary step for a bacterium to become widespread and successful under the selective pressure of antibiotics in hospital environment. However, maintaining resistant phenotype and carrying various resistance

genes in the absence of selective pressure imposes a cost in the fitness (capability to survive and to reproduce) of the bacterium. For example, carriage of certain plasmids may reduce the fitness of bacteria by >5% per generation (63, 64). Mutations leading to resistance may impair the fitness of bacteria because they target important biological functions of the cell (65). Antibiotic resistance associated with high fitness cost may become contra-selective for the success of resistant strains. For example, high level resistance to vancomycin is associated with such high fitness cost in MRSA that it prevented the widespread dissemination of VRSA strains (66). Differences in resistance and associated level of fitness may attribute to the varying success of different clones of MRSA.

1.8. Differences between MRSA and MSSA strains

MRSA most importantly differ from MSSA strains in their β-lactam resistance, however, this is not their only dissimilarity. MRSA strains carry the SCC*mec* genetic element, that most MSSA lack, and besides *mec* genes, several other antibiotic resistance and regulatory genes are encoded on this cassette. Because of the presence of SCC*mec* and other acquired resistance mechanisms, MRSA are often multi-resistant to antibiotics of different classes, whereas MSSA strains are generally more sensitive to non-β-lactam antibiotics as well (67).

Virulence of the pathogen and outcome of infection are, however, difficult to compare between MRSA and MSSA isolates. Most studies report increased mortality rate in patients with MRSA infections (68-70). Some other investigations debate this and report comparable mortality in MSSA bloodstream infection (BSI) suggesting that adjustment to confounding factors, as comorbidities, age and severity of illness, delayed initiation of effective therapy may nullify the impact of resistance on the outcome of the infection (71-73). Some studies even suggest that MSSAs may cause more severe infections, supposed to be related to higher prevalence of virulence genes in MSSA or to the greater fitness cost associated with SCC*mec* cassette in MRSA (74).

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2. OBJECTIVES

The objectives of our study were the following:

(1) To examine the possible role of fluoroquinolone resistance in the varying fitness of different clones of MRSA, as a potential contributor to the emergence and success of new epidemic clones.
(2) To compare the antibiotic susceptibility, virulence factors and associated mortality in MRSA and MSSA BSI infections.
(3) To evaluate the clonal composition of MRSA isolates currently causing BSI at the Semmelweis University Clinics and to compare characteristics of isolates of different MRSA clones.

3. RESULTS

3.1. Fitness cost associated with fluoroquinolone resistance in different MRSA clones

3.1.1. Fitness of ciprofloxacin resistant mutants of CA-MRSA strains

To determine the fitness cost of fluoroquinolone resistance, we induced ciprofloxacin resistance in various clones of CA-MRSA by exposing the strains to increasing concentrations of the antibiotic. Changes in the fitness of the bacteria was compared in propagation assay by measuring the growth rate of the isogenic ciprofloxacin sensitive strains and their multiple ciprofloxacin resistant derivates in monocultures (Figure 5). Growth rate was determined by measuring optical density (OD) values. Area under the curve (AUC) was used for the numerical comparison of the growth capacity of the strains, higher AUC values indicating faster replication rates. Identity of the wild type CA-MRSA strains with their ciprofloxacin resistant variants was confirmed by PFGE.

As the result of exposure to ciprofloxacin, the originally ciprofloxacin sensitive CA-MRSA strains gained mutations in the genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*grlA*, *grlB*), detected by sequencing the respective genes (Table 3) (75). No genetic alteration in the *gyrB* gene could be detected. Strain 3 (ST30-IV) carried several mutations in the *grlB* gene that were present in the original, ciprofloxacin sensitive variant of the strain, as well. These mutations were the following: Asp420→Asp (AAC→AAT)+Thr421→Thr(ACT→ACA)+Glu422→Asp(GAA→GAT)+Leu464→Leu(CTA→CTG). These mutations did not seem to influence ciprofloxacin MIC values alone, however, this combination of genetic alternations may have contributed to the sudden rise of ciprofloxacin MIC in this strain when exposed to the antibiotic. The Ser80→Phe mutation in the *grlA* gene seemed to severely compromise the fitness of ciprofloxacin resistant derivatives of ST8-IV and ST80-IV isolates (Table 3). Each ciprofloxacin resistant CA-MRSA strain variant grew much slower than their respective ciprofloxacin sensitive ancestor (Figure 5). However, development of ciprofloxacin resistance influenced the speed of replication to different extent in the different CA-MRSA clones. Derivatives of ST30-IV (strain 3) grew the slowest. The ST8-IV (strain 1)

derivative with 256 mg/L MIC value suffered greater loss of speed than the ST80-IV (strain 2) derivative with the same MIC (23.7 vs 33.5 AUC, respectively) (Figure 5, Table 3).

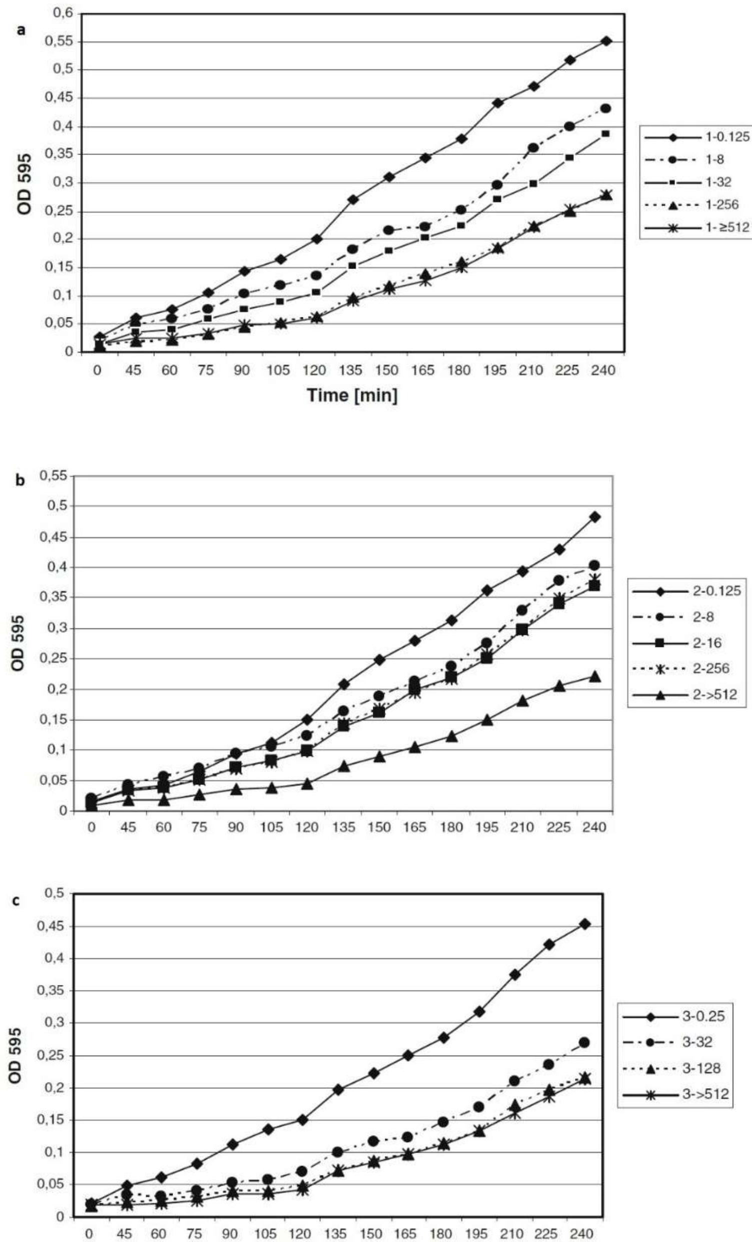

Figure 5. Growth curves of CA-MRSA strains and their ciprofloxacin resistant derivatives. (a) Strain 1: ST8-IV (b) Strain 2: ST80-IV (c) Strain 3: ST30-IV (Legend of the graphs indicates the strain number and the MIC value to ciprofloxacin)

Table 3. Antibiotic resistance profiles, AUC values and genetic alterations in the QRDR regions of the *gyrA* and *grlA* genes in the wild type CA-MRSA strains and their ciprofloxacin resistant derivatives

Strain	Clone/ sequence type	Ciprofloxacin MIC (mg/L)	AUC	*GyrA* mutation	*GrlA* mutation
1	**ST8-IV**	0.125	57.8	–	–
		8	41.5	–	Ser80→Phe (TCC→TTC)
		32	34.9	–	Ser80→Phe (TCC→TTC)
		256	23.7	Glu88→Lys (GAA→AAA)	Ser80→Phe (TCC→TTC) + Glu84→Lys (GAA→AAA)
		>512	23.5	Glu88→Lys (GAA→AAA)	Ser80→Phe (TCC→TTC) + Glu84→Lys (GAA→AAA)
2	**ST80-IV**	0.125	44.9	–	–
		8	38.16	–	Ser74→Tyr (TCT→TAT)
		16	33.35	–	Ser74→Tyr (TCT→TAT)
		256	33.54	Ser84→Leu (TCA→TTA)	Ser74→Tyr (TCT→TAT)
		>512	18.6	Ser84→Leu (TCA→TTA)	Ser74→Tyr (TCT→TAT)
3	**ST30-IV**	0.25	44.4	–	–
		32	23.7	Ser84→Leu (TCA→TTA)	Ser80→Phe (TCC→TTC)
		128	18.6	Ser84→Leu (TCA→TTA)	Ser80→Phe (TCC→TTC)
		>512	17.7	Ser84→Leu (TCA→TTA)	Ser80→Phe (TCC→TTC)

3.1.2. Fitness of ciprofloxacin resistant HA-MRSA isolates

To gain insight into the background of the successful spread of the most predominant MRSA clones spreading in Hungarian hospitals, we determined the fitness of representatives of the following clones: ST239-III (Hungarian/Brazilian), ST228-I (South-German), ST5-II (New York – Japan), ST22-IV (EMRSA-15). Antibiotic resistance, ciprofloxacin MIC values and AUC representing the speed of growth are summarized in Table 4. ST239-III isolates replicated much slower compared to strains from other clones. Overall ST22-IV isolates had the highest growth rate, and could combine the fastest replication with the highest ciprofloxacin MIC values (Figure 6).

Table 4. Antibiotic resistance profiles and AUC values of HA-MRSA strains from different clones

Strain	Clone	CIP MIC (mg/L)	Antibiotic resistance	AUC
1	**ST228-I**	128	Ox, Cn, Da, E, Cip	39.4
2	**ST22-IV**	256	Ox, Da, E, Cip	52.4
3	**ST22-IV**	256	Ox, Cip	39.0
4	**ST5-II**	128	Ox, Cn, Da, E, Cip, Sxt, Rif	34.0
5	**ST5-II**	64	Ox, Da, E, Cip	52.9
6	**ST239-III**	32	Ox, Cn, Da, E, Cip, Tc, Sxt	26.3
7	**ST239-III**	4	Ox, Cn, Da, E, Cip, Tc	35.1

Kn kanamycin, Cip ciprofloxacin, Fc fusidic acid, Cn gentamicin, Da clindamycin, Tc tetracycline, Sxt trimethoprim/ sulphametoxazole, Rif rifampicin

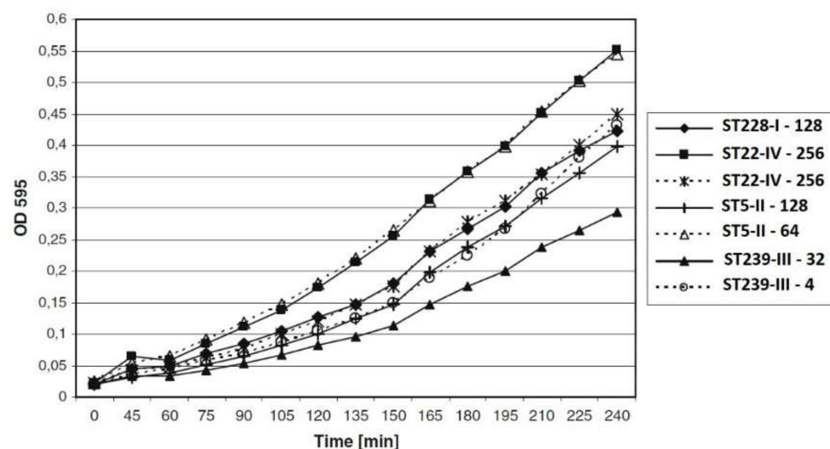

Figure 6. Growth curves of HA-MRSA strains representing the most prevalent MRSA clones (The figure legends show the clonal type and ciprofloxacin MIC value (mg/L))

3.2. Characterisation of MRSA and MSSA isolates of bloodstream infection (BSI)

3.2.1. Origin and baseline characteristics of the study population

To compare the antibiotic susceptibility, virulence factors and associated mortality in MRSA and MSSA BSI infections; to evaluate the current predominant MRSA clones in Hungary; and to compare characteristics of isolates of different MRSA clones, we studied the strain collection of the Institute of Laboratory Medicine, Semmelweis University, Budapest, Hungary (76).

All non-duplicated BSI MRSA strains isolated between January 2011 and December 2016 at the laboratory, overall 153 MRSA isolates were included in the study. Each year, the same number of MSSA BSI isolates, representing the same gender and age distribution of population and hospital wards were enrolled (from a much larger pool) to be compared to the MRSA strains. In total, 306 *S. aureus* BSI isolates (153 MRSA and 153 MSSA strains) were analysed. From patient related factors, gender, age, comorbidities, current chemotherapy and steroid therapy was registered. Charlson comorbidity index (CCI) and all-cause 30 days mortality were determined for each patient (Table 5). Of the patients with MRSA BSI, significantly more were males than females (61.4% vs 38.6%, p=0.044), MSSA isolates were selected to match this ratio. CCI was significantly higher in female patients (4.92 vs 4.24 in males, p=0.0164). Chronic liver disease and chemotherapy was more frequent in MSSA patients, whereas more of MRSA patients had surgery in the previous 30 days or endocarditis, however, CCI did not differ significantly in the two groups.

Table 5. Characteristics of *S. aureus* BSI patients

	MSSA n=153		MRSA n=153		*p*
Age (median, range)	64 (0-94)		68 (0-98)		
	n	%	n	%	
Male sex	93	60.08	94	61.4	0.90665
Diabetes	48	31.4	57	37.3	0.2785
Chronic liver disease	**33**	**21.6**	13	8.5	**0.0014**
Chronic kidney disease	26	17.0	21	13.7	0.4279
Solid tumor	39	25.5	25	16.3	0.0491
Haematology malignancy	20	13.1	15	9.8	0.3691
Chemotherapy	**21**	**13.7**	4	2.6	**0.0006**

Steroid treatment	14	9.2	9	5.9	0.2783
Surgery in previous 30 days	34	22.2	**54**	**35.3**	**0.0115**
Endocarditis	3	2.0	**12**	**7.8**	**0.0172**
Charlson comorbidity index (mean)	4.65		4.36		0.72634

3.2.2. Antibiotic susceptibility of MRSA and MSSA isolates

MRSA isolates were more resistant to all antibiotics except for doxycycline (Figure 7). Multidrug resistance (MDR) was also significantly more prevalent in MRSA isolates, whereas 75.8% of MSSA isolates were susceptible to all tested antibiotics.

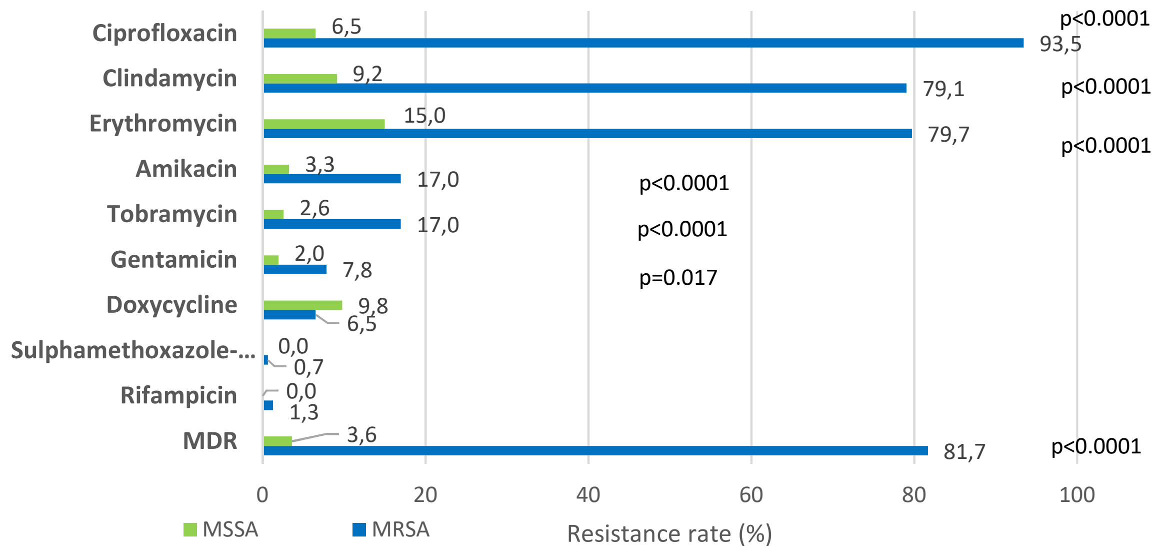

Figure 7. Antibiotic susceptibility and multidrug resistance of MRSA and MSSA isolates

All MRSA and MSSA isolates were sensitive to vancomycin, teicoplanin and linezolid. Vancomycin MIC was 2 mg/L in 7.8% of isolates. Vancomycin MIC_{50} values increased from 0.5 mg/L in 2011-2012 to 1 mg/L in 2013-2016. Six point five percent of the isolates had teicoplanin MIC=2 mg/L.

3.2.3. Virulence factors of MRSA and MSSA isolates

MRSA strains carried a median of six virulence genes. The most frequent virulence type in MRSA carried genes encoding *hla, hlb, hlg, ica, spa, cna*, and *sea* or *seb* (11.1% and 14.4% of the isolates, respectively). Isolates were highly diverse; we identified 57 different virulence gene combinations in MRSA isolates. MSSA strains carried less virulence factors (median of 5). Most frequent virulence type in MSSA was *hla, hlb, hlg, hlgv, ica, spa* positivity.

Cna, sea, ica and *hlb* were significantly more prevalent in MRSA, whereas *tst, eta, sec* and *hlgv* were significantly more frequent in MSSA (Table 6). Superantigens were more frequent in MSSA, whereas adhesins were more frequent in MRSA isolates. *LukS-PV/lukF-PV* positivity rate was 3.3% and 1.3% in MRSA vs MSSA, respectively. The prevalence of this gene changed significantly during the 6 years of the study: it was 13% in MRSA isolates in 2011, but never exceeded 4% in the later years.

Table 6. Virulence factors of MRSA and MSSA isolates

Virulence genes		MRSA		MSSA		All		p values
		n	%	n	%	n	%	
Superantigens	***tst***	0	0,0	**4**	**2,6**	4	**1,3**	**0.044**
	eta	0	0,0	**4**	**2,6**	4	**1,3**	**0.044**
	etb	1	0,7	3	2,0	4	**1,3**	0.3141
	sea	**30**	**19,6**	17	11,1	47	**15,4**	**0.0390**
	seb	58	37,9	49	32,0	107	**35,0**	0.2806
	sec	12	7,8	**25**	**16,3**	37	**12,1**	**0.0223**
Cytotoxins	***lukS-PV/ lukF-PV***	5	3,3	2	1,3	7	**2,3**	0.2513
	hla	111	72,5	118	77,1	229	**74,8**	0.3564
	hlb	**106**	**69,3**	75	49,0	181	**59,2**	**0.0003**
	hlg	89	58,2	84	54,9	173	**56,5**	0.5642
	hlg-v	31	20,3	**93**	**60,8**	124	**40,5**	**<0.0001**
Adhesins	***icaA***	**122**	**79,7**	85	55,6	207	**67,6**	**<0.0001**
	spa	150	98,0	152	99,3	302	**98,7**	0.3141
	cna	**110**	**71,9**	45	29,4	155	**50,7**	**<0.0001**

All but one MRSA and one MSSA isolates carried at least one adhesion factor encoding gene (*ica*, *spa* or *cna*). The majority of isolates possessed at least one cytotoxin gene (*pvl, hla, hlb, hlg*, or *hlgv*). Compared to cytotoxins, significantly less isolates were positive for superantigens. Approximately half of the isolates carried enterotoxin encoding genes (*sea, seb* or *sec*), and only a low number of isolates had toxic shock syndrome toxin or exfoliative toxin (Figure 8).

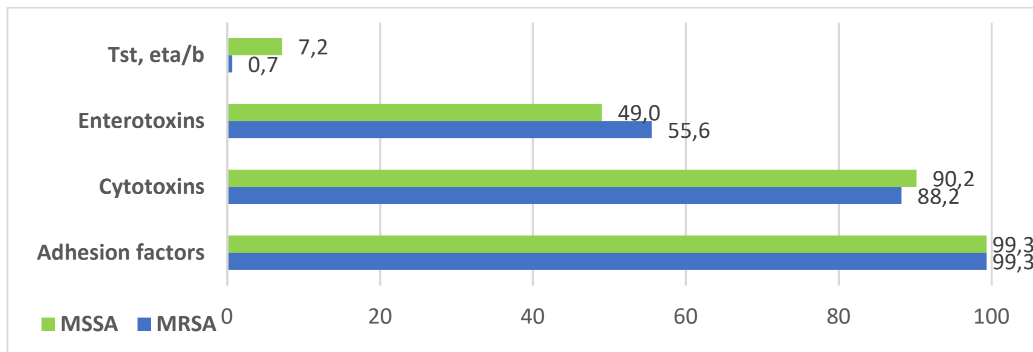

Figure 8. Percentage of isolates carrying virulence genes (%)

3.2.4. Clonal diversity of the isolates

Our MSSA strains showed high level of diversity based on the PFGE comparison, no dominant clone was identified. On the contrary, most of the MRSA isolates belong to 3 main PFGE pulsotypes (Figure 9a and 9b).

SCC*mec* typing of the MRSA isolates showed that the vast majority of our strains belonged to SCC*mec* type IV. SCC*mec* type III and VI isolates were not found, and only one of the isolates from 2013 had SCC*mec* type V. SCC*mec* IV isolates kept their dominance throughout the 6 years of the study (Figure 10). SCC*mec* type IV isolates were significantly more frequent in females (78.0% vs 59.6% in males, p=0.0188).

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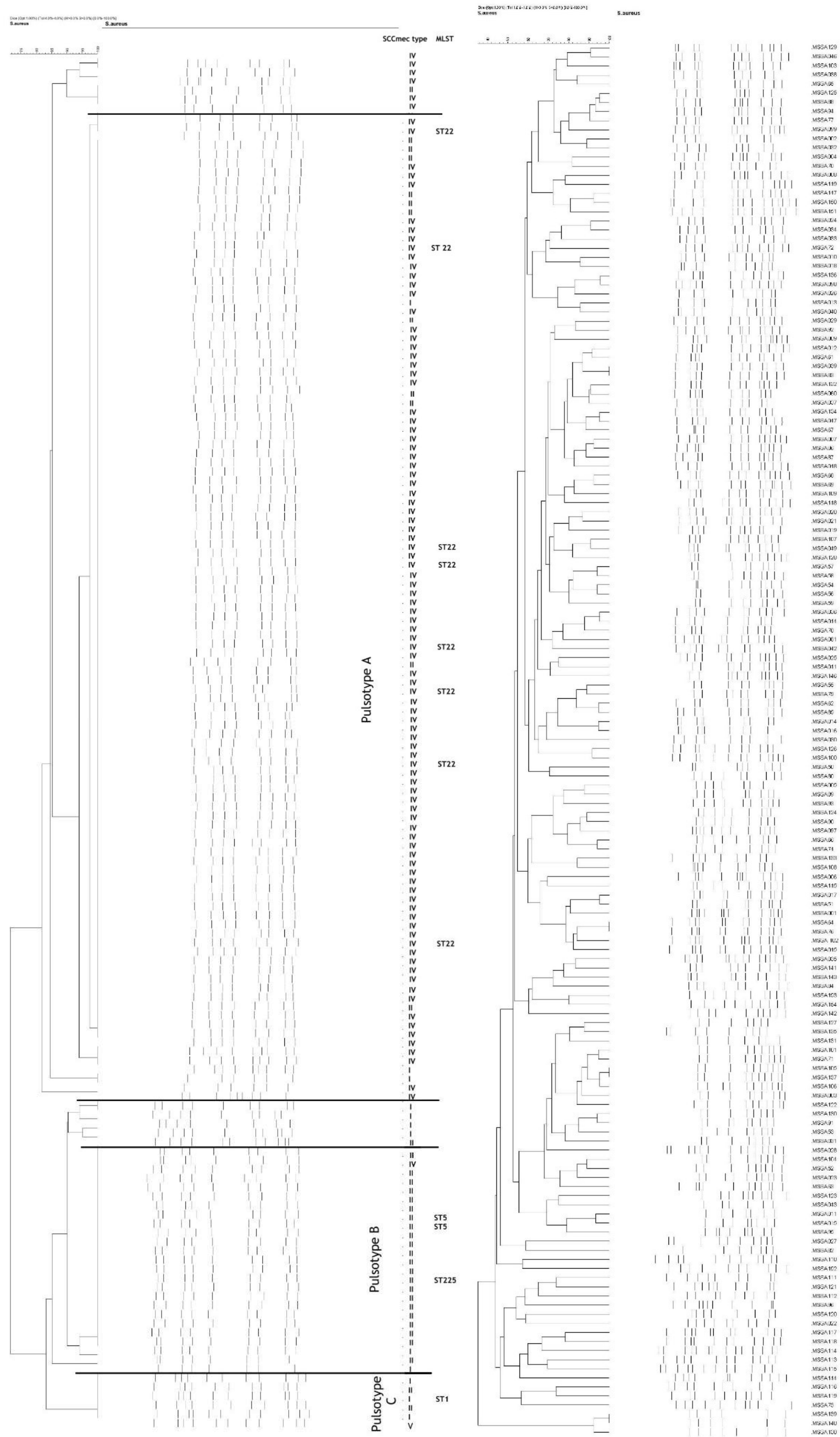

Figure 9. PFGE pulsotype of the MRSA (a) and the MSSA (b) isolates

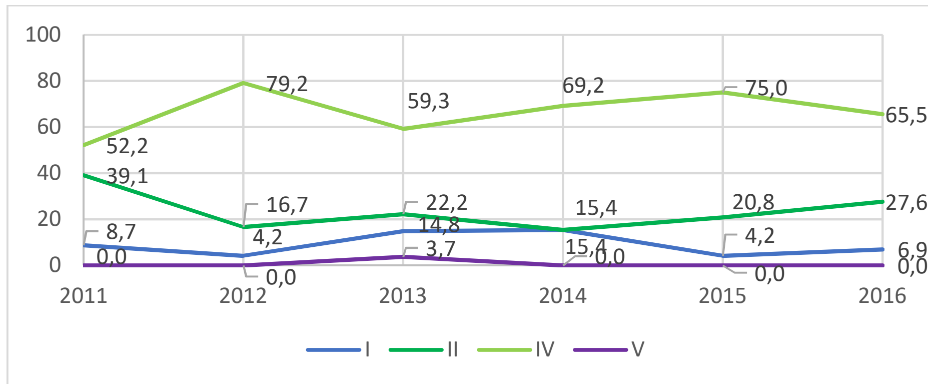

Figure 10. Changes in the prevalence of different SCC*mec* types among MRSA isolates (%)

MLST was carried out for 12 representative MRSA isolates from the most frequent PFGE pulsotypes and SCC*me*c types, representing all six years of the study. All eight tested SCC*mec* IV, PFGE type A isolates belonged to the ST22 clone. Three SCC*mec* II, PFGE type B isolates were typed: two belonged to ST5 and one to ST225, the latter being also part of CC5. Our representative SCC*me*c type I, pulsotype C isolate belonged to ST1.

3.2.5. Differences between MRSA isolates of different clones

MRSA isolates had a number of differences compared to MSSAs. Moreover, significant differences were found in the antibiotic resistance, virulence and associated mortality among MRSA isolates of different clones.

SCC*mec* II isolates were associated with especially high resistance rates to gentamycin, amikacin, tobramycin and doxycycline (Table 7). The highest vancomycin MIC values were observed in this group. SCC*mec* IV isolates were relatively less resistant to antibiotics compared to the other two groups, except for ciprofloxacin.

SCC*mec* II isolates carried the highest number of virulence factors. Panton-Valentine leukocidin gene was found exclusively in SCC*mec* I and II isolates. Our sole SCC*mec* V isolates did not carry any of the tested virulence factor genes.

Table 7. Antibiotic resistance and high vancomycin MIC values in MRSA isolates of different SCC*mec* types

	SCC*mec* I n=14		**SCC*mec* II n=36**		**SCC*mec* IV n=102**		**MRSA all n=153**	
	R	**%**	**R**	**%**	**R**	**%**	**R**	**%**
Erythromycin	13	92.9	33	91.7	75	73.5	122	79.7
Clindamycin	13	92.9	32	88.9	75	73.5	121	79.1
Gentamicin	6	42.9	3	8.3	3	2.9	12	7.8
Tobramycin	7	50.0	12	33.3	7	6.9	26	17.0
Amikacin	7	50.0	12	33.3	7	6.9	26	17.0
Ciprofloxacin	11	78.6	35	97.2	97	95.1	143	93.5
Co-trimoxazole	0	0.0	0	0.0	1	1.0	1	0.7
Doxycycline	3	21.4	1	2.8	5	4.9	10	6.5
Rifampicin	1	7.1	0	0.0	1	1.0	2	1.3
Vancomycin MIC=2mg/L	3	21.4	4	11.1	4	3.9	10	6.5

Table 8. Virulence factors in MRSA isolates of different SCC*mec* types

	SCC*mec* I n=14		**SCC*mec* II n=36**		**SCC*mec* IV n=102**		**MRSA all n=153**	
	R	%	R	%	R	%	R	%
tst	0	0.0	0	0	0	0	0	0%
eta	0	0.0	0	0	0	0	0	0%
etb	0	0.0	1	**2.8**	0	0	1	0.7%
sea	6	**42.9**	11	**30.6**	13	12.7	30	19.6%
seb	5	35.7	12	33.3	41	**40.2**	58	37.9%
sec	0	0.0	4	**11.1**	8	7.8	12	7.8%
lukS-PV/lukF-PV	2	**14.3**	3	**8.3**	0	0.0	5	3.3%
hla	10	71.4	29	**80.6**	72	70.6	111	72.5%
hlb	8	57.1	23	63.9	75	**73.5**	106	69.3%
hlg	5	35.7	22	**61.1**	62	**60.8**	89	58.2%
hlg-v	10	**71.4**	19	**52.8**	2	2.0	31	20.3%
icaA	11	78.6	28	77.8	83	**81.4**	122	79.7%
spa	14	**100.0**	36	**100.0**	100	98.0	150	98.0%
cna	5	35.7	12	33.3	93	**91.2**	110	71.9%
Median n of virulence genes	5.5		7		6		6	

3.2.6. Differences in the mortality in BSI caused by different *S. aureus* types

Although CCI of the patients did not differ significantly, BSI caused by MRSA led to significantly higher mortality rates (39.9% vs 30.7% in MSSA BSI, respectively, $p<0.0001$). Overall 30-day mortality was 35.3%.

Mortality was significantly higher in females (38.7% vs 33.2% in females and males, respectively ($p<0.001$)), this could be attributed to higher CCI of the female patients in this study. Mortality increased with age: it was 20.0% in the age group 0-49y, 28.0% in 50-64y, 40.2% in 65-79y and 55.8% in patients older than 80 years.

Higher vancomycin MIC did not influence mortality rates. Although we have found only 10 isolates with teicoplanin MIC of 2 mg/L, mortality was 70% in this group.

Number of carried virulence genes, presence of specific virulence factors and antibiotic resistance to other drugs besides glycopeptides did not influence mortality.

Mortality rates differed in BSI caused by MRSA isolates of different clones (Table 9). Interestingly, patients infected with SCC*mec* IV isolates had higher mortality, than patients infected with SCC*mec* I or II MRSA strains (42.2% in SCC*mec* IV vs 28.6% and 36.1% in SCC*mec* I and II, respectively), however, these differences were not statistically significant. CCI of patients infected with MRSA isolates of SCC*mec* I and II group did not differ significantly when compared to CCI of SCC*mec* IV group.

Table 9. Mortality rates (%) and CCI of patients in BSI caused by different types of *S. aureus*

	MRSA-SCC*mec* I	**MRSA-SCC*mec* II**	**MRSA-SCC*mec* IV**	**MSSA**
Charlson comorbidity index	4.29	3.97	4.46	4.65
Mortality rate (%)	28.6	36.1	42.2	30.7

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4. DISCUSSION

4.1. Impact of fluoroquinolone resistance on the fitness of MRSA isolates of different clones

4.1.1. Differences in the fitness of CA-MRSA and HA-MRSA isolates

Propagation assay clearly showed that development of fluoroquinolone resistance has a great impact on the fitness (speed of growth) of CA-MRSA strains.

Extent of loss of fitness was directly influenced by the number of mutations in the QRDR regions of the fluoroquinolone resistance genes. Amino acid substitutions in this region, especially at the *grlA* Ser80 position have provided the strains the ability to gain resistance to fluoroquinolones, however at a serious price, as it has greatly compromised the vitality of strains (Table 3). Even silent nucleotide substitutions in the *grlB* gene that did not change the amino acid sequence could result in loss of speed of replication, as they can affect protein folding and can slow down translation because of differences in the availability of tRNA in the cell (77, 78).

Level of fitness cost was different in isolates of various MRSA clones. Fluoroquinolone resistant derivatives of all three CA-MRSA strains showed much slower replication rates compared to the originally ciprofloxacin resistant HA-MRSA isolates. This observation can explain why these CA-MRSA clones were less capable of spreading in hospital setting than strains of HA-MRSA clones. CA-MRSA strains can adapt to fluoroquinolones, but in the process they suffer so great loss of fitness, that the strains with higher replication rates will overgrow and outcompete them from the niche.

On the other hand, these CA-MRSA strains will be more successful than HA-MRSA outside the healthcare system where resistance to fluoroquinolones is not an asset. From the studied HA-MRSA clones, only one of the ST22-IV (Strain 5) and one of the ST5-II (Strain 13) isolates retained sufficient fitness despite high ciprofloxacin MIC values to potentially compete with the replication rate of the wild type ST80-IV and ST30-IV CA-MRSA isolates (Tables 3 and 4). When comparing the original, non-mutated form of the studied isolates, the ST8-IV (Strain 1) CA-MRSA grew with the highest replication rate

(characterised by AUC of 57.8) of all the isolates, therefore in the community, in fluoroquinolone free setting this strain would likely have an advantage over all other strains.

4.1.2. Differences in the fitness of the predominant HA-MRSA clones and their effect on the clonal replacement in Hungary

Our study also revealed differences in the fitness cost of HA-MRSA isolates of different clones. These observations can provide an explanation for the dynamics of clonal replacement observed in Hungary in the previous decades. In our study, isolates of ST239-III (Hungarian-Brazilian) clone grew the slowest, despite relatively lower ciprofloxacin MIC values compared to other HA-MRSA strains (Table 4). Isolates of this clone were dominant in Hungary in the 1990s, and in this period incidence of MRSA was low (56). From 2000, ST239-III clone was gradually replaced by ST5-II (New York – Japan) and ST228-I (South-German) clones, and this clonal replacement was coupled by a steep rise in the incidence of MRSA among invasive *S. aureus* isolates between 2000 and 2006 (Figure 11). In 2007 and 2008, MRSA incidence levelled off. As our results indicate, isolates of ST5-II and ST228-I clones are capable of maintaining much higher replication rates in spite of higher levels of ciprofloxacin MIC values, and this could provide a plausible explanation for their successful replacement of the previously dominant ST239-III clone.

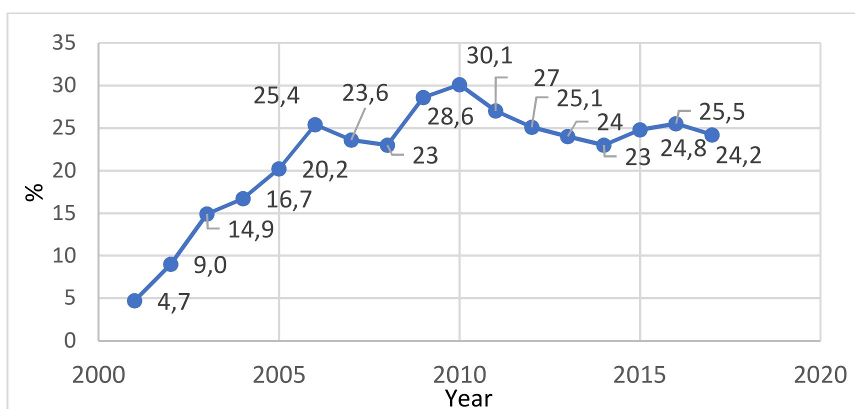

Figure 11. Prevalence (%) of MRSA among invasive isolates of *S. aureus* in Hungary (75, 79)

From 2008, ST22-IV gradually became to most prevalent clone in Hungary, its ratio among MRSA was nearly 60% in 2013 (personal communication from Ákos Tóth, National Public Health Center of Hungary). The clonal replacement of ST5-II and ST228-I types led to the rise of MRSA incidence between 2008 and 2010, followed again by levelling off and decrease of the incidence in the subsequent time period without the evidence of a new major HA-MRSA clone invading the country.

In our study, ST22-IV strains proved to be much fitter than the ST239-III isolates. Moreover, one ST22-IV isolate (strain 5) showed comparable level of fitness to that of the most fit ST5-II isolate (strain 13), despite having much higher ciprofloxacin MIC, and expressed significantly higher vitality than the other ST5-II isolate (strain 9) and the ST228-I isolate. Our second ST22-IV isolate (strain 8) had higher ciprofloxacin MIC, higher AUC than one of the ST5-II and identical AUC to the ST228-I isolate. This overall shows the superiority of ST22-IV isolates in the terms of fluoroquinolone resistance and vitality over ST5-II and ST228-I isolates, and can explain the replacement of these previous clones around 2010, similarly to the way they replaced ST239-III clone a decade earlier. The fitness difference, however, was smaller between ST22-IV and ST5-II and ST228-I, than that of those over ST239-III. This can explain why the ST239-III clone was replaced quickly and entirely by ST5-II and ST228-I, whereas ST22-IV replaced these more gradually, and not entirely in the case of ST5-II.

4.1.3. Clonal replacement on the global scale

Similar clonal replacements of *S. aureus* were observed all over the world in the past decades. The first international HA-MRSA clone, ST5-II disseminated in the 1990s all over the world. Subsequently ST22-IV, an originally CA-MRSA strain that became successful in hospitals too, emerged and replaced the previously successful clones in many countries (57-62). According to a German study, ST5-II isolates replaced the originally predominant ST228-I (South-German) and ST45-IV (Berlin MRSA) clone around 2000, to be later succeeded by ST22-IV by 2006 (80). In South-East Austria, ST228-I (South-German) and ST8-II (Irish) clone was prevalent in 2002, and was replaced by ST5-II in 2012. Incidence of ST22-IV started to rise in the same year (81). In

Singapore, ST22-IV have supplanted the previously dominant ST239 clone in 2010 (82). In an Italian study, ST22-IV replaced ST228-I as the dominant clone in 2008 (83).

Fluoroquinolones were introduced to the clinical use in the late 1990s in many countries, and their consumption rose during the 2000s (84). As it was posed by Füzi et al., widespread use of fluoroquinolones in the healthcare could have been the force that selected the first HA-MRSA strains, the ST5-II clones of *S. aureus* globally, on the basis of their ability to maintain their fitness and resistances to fluoroquinolones at the same time, unlike ST30, ST8 and ST239 (84). Later the ST22-IV clone surpassed the ST5-II in success of dissemination, possibly explained by its even higher replication rate combined with even higher fluoroquinolone MIC values (75, 84).

Although use of other antibiotics besides fluoroquinolones may also influence changes in the dissemination of *S. aureus* (85, 86), impact of these antibiotics on the success of major MRSA clones remains secondary (84).

Pathogenicity and virulence of MRSA clones have also been studied in relation to the clonal dynamics of the pathogen.

Panton-Valentine leukocidin (PVL), an important virulence determinant of *S. aureus* is mostly produced by antibiotic susceptible, small-size SCC*mec* CA-MRSA isolates. It seems that major HA-MRSA clones do not need the extra virulence provided by PVL for their success, moreover, fitness-gain associated with the lack of production of PVL may help them disseminate in hospital setting (84).

Presence of ACME was shown to have a role in the success of ST8 (USA300) clone. However, ACME is missing from strains of novel lineages of ST8 MRSA clone disseminating in hospital settings on different continents (87). This suggests that ACME, although enhances the survival of the pathogen on the skin of patients, is not essential for the successful spread of the clone (84).

Although biofilm production may increase the capacity of ST22-IV clone to disseminate, this may not be the sole reason for its success, as ST22-IV isolates are generally inferior in biofilm production to the strains of ST228 and ST8 clones (83), which they can easily replace in hospital setting.

Biofilm production and other virulence factors are more likely to increase the success of a given clone in fluoroquinolone-free settings, as in the community or at paediatric wards (84).

Since fluoroquinolones have such an important role in selection of major MRSA clones, decrease in the use of fluoroquinolones could result in decline of the prevalence of these major clones. According to a recent survey extended to seven countries, policies to reduce MRSA rates, including limited use of cephalosporins and fluoroquinolones were associated with reduction in MRSA incidence (88). In the United Kingdom, fluoroquinolone consumption in the hospital sector was reduced by almost 50% between 2005 and 2012 (89). During the same period, rate of MRSA among *S. aureus* isolates causing invasive infections declined from 43.6% to 14.0% in the country (90). Judicious use of fluoroquinolones could result in reduction in the rates of HA-MRSA and other MDR pathogens (84, 88, 89).

4.1.4. Role of fitness cost of fluoroquinolone resistance in the clonal replacement in other bacteria

Similarly to *S. aureus*, globally successful large clones of many other multidrug resistant bacteria emerged over the last 2-3 decades. Varying fitness cost associated with fluoroquinolone resistance may have played a role in the selection of these dominant clones as well. Growth advantage of the dominant clones over minor clones were demonstrated for ESBL-producing *Klebsiella pneumoniae* (91), ESBL producing *Escherichia coli* (92, 93), and *Clostridioides difficile* (94). Effect of fitness cost on the selection of international clones of different MDR pathogens was reviewed by Füzi et al. (84, 95, 96).

4.2. Differences between various types of *S. aureus* isolates causing BSI

S. aureus is a major cause of BSI in hospital settings, associated with high mortality. MRSA was the most frequent multidrug resistant organism causing hospital acquired BSI in Hungary in 2010 (97). However, to our knowledge, our study was the first to describe

antibiotic resistance, virulence factors and current clonal composition of *S. aureus* BSI isolates from Hungary (76) .

4.2.1. Differences between MRSA and MSSA

Importance of methicillin resistance of *S. aureus* in the severity and in the outcome of the infection is a long debated topic in the literature.

In our study, we have found higher mortality rates in patients with MRSA infections compared to BSI caused by MSSA isolates (Table 9). Older age, female gender, infection with SCC*mec* type IV MRSA and high teicoplanin MIC value of the isolate were associated with worse outcome of the infection.

MRSA isolates had significantly higher resistance rates and more frequent multidrug resistance compared to MSSA (Figure 7). MRSA were significantly more resistant to ciprofloxacin, erythromycin, clindamycin, amikacin, tobramycin and gentamicin; the only antibiotic with higher resistance rate in MSSA was doxycycline. Higher antibiotic resistance rates towards multiple antibiotics may provide an explanation for high mortality ratio in MRSA infections. In septic patients, delayed administration of effective antibiotic therapy greatly increases mortality (98). Ineffective initial empirical therapy is described to be more frequent in patients with MRSA BSI (99).

Virulence gene patterns were also different in MRSA and MSSA isolates in our study (Table 6, Figure 8). MRSA isolates carried more virulence genes, however, number of carried virulence genes or presence of any specific virulence factor did not influence the outcome of the infections. Particularly adhesion factors (*cna* and *ica*) were significantly more prevalent in MRSA, whereas genes encoding superantigens (especially *sea*, *eta* and *tst*) were more prevalent in MSSA.

4.2.2. Antibiotic sensitivity

4.2.2.1. MRSA rates

In our laboratory, MRSA rates among *S. aureus* BSI isolates varied between 27.5% and 40.7% during the 6 years of the study. These figures are higher than the national average

(between 23.0% and 27.0% in the same period, Figure 11). Different disease severity and patient population in university clinics may provide an explanation for this observation.

4.2.2.2. Glycopeptide susceptibility

In our study, all MRSA isolates were sensitive to glycopeptides, however, vancomycin MIC values seemed to creep higher from 2011 to 2015, with a slight decrease in 2016. 'Vancomycin creep', i.e. gradual increase of the proportion of MRSA isolates with high glycopeptide MIC, is a controversial topic in the literature. Several studies report increase in MIC values over time, while others did not confirm these findings (100). Some authors suggest vancomycin creep may be a regional problem, that may occur in a certain area or hospital, and not seen in others (101).

The possibility of gradual increase in vancomycin MIC requires special attention as it might lead to development of resistant strains, and poorer clinical outcome was reported in patients infected with isolates exhibiting higher glycopeptide MIC values (100). In our study, increased vancomycin MIC was not associated with more severe outcome. However, although we have found only 10 isolates with high teicoplanin MIC (2 mg/L), the mortality was very high in this specific group (7 out of 10 patient died).

4.2.2.3. MRSA and gender

We have found that from those patients, who had MRSA positive blood culture sample, significantly more were males (61.4%), than females (38.6%) ($p=0.044$). Male dominance in both carriage and BSI MRSA infections was described in several studies, for example, in 5 independent studies from 20 centres, males were statistically overrepresented in MRSA positive samples in all 5 studies, accounting for 54.7%–66.6% of patients (102). Humpfreys et al. suggest the possible role of hand-hygiene behaviour, oestrogen, contact sports and occupation behind this gender difference. They also mention that although males are more prone to bacterial sepsis, some studies suggest poorer outcome of sepsis in females (103). This is in concordance with our findings, we found significantly higher mortality rate in female than in males (38.7% vs 33.2%, $p<0.001$), however, CCI of female patients was also higher. Other studies have found that

the association between gender and *S. aureus* BSI mortality may be influenced differently by the severity of comorbidities in males and female s (104).

4.2.3. Virulence

Several studies have found low prevalence of PVL in *S. aureus* isolates from BSIs, and described it to be more closely associated with skin and soft tissue infections (23, 74). In our study, we found low *pvl* rate (2.3% for all samples), and *pvl* was more frequent in MRSA isolates. In a recent study on BSI MSSA, declining rates of *pvl* and other virulence genes were observed (74). This is in concordance with our findings in MRSA isolates: in 2011, 13.0% of our MRSA isolates were positive for *pvl,* yet in the following years *pvl* prevalence remained below 4%. *Tst* was found exclusively in MSSA. For Staphylococcal protein A (*spa*), 98.7% of our isolates were positive. It is described that about 1-2% of *S. aureus* isolates carry rearrangements in the IgG-binding region of the *spa* gene, thus rendering them non-typable with spa-typing (105). Genetic changes in the *spa* gene may also lead to negativity in Staphaurex test, making the presumptive identification of these strains more difficult (106).

Number of carried virulence genes and presence of any specific virulence factor did not influence the outcome of the infection.

Several studies described the role of specific *S. aureus* virulence determinant in establishment of an infection and in severity of the disease (107). Nevertheless, our findings suggest that outcome of the infection is more related to antibiotic resistance and patient related factors such as age and gender than virulence determinants of the bacteria.

4.2.4. Clonal diversity

It is well established that successful *S. aureus* clones are competing and replacing their rivals, changing the clonal map over time. In North and South America and in Japan, ST8-IV (USA 300) MRSA is the dominant clone, while in Europe and Asia ST239-III (Hungarian-Brazilian) strains are being replaced by ST22-IV clone (also known as EMRSA-15) (108).

In our study, the majority (66.7%) of the BSI MRSA isolates belonged to the ST22- IV type. SCC*mec* IV isolates were previously considered as generally community-acquired MRSA, however, this clone became widespread and successful in hospital settings, too (11). Despite slight changes in prevalence, SCC*mec* IV isolates kept their dominance in our samples throughout the 6 years of the study (Figure 10).

Our SCC*mec* II isolates belonged to clonal complex 5 (CC5). ST5-II MRSA and its single locus variant, ST225-II were both found. ST5-MRSA-II (New-York –Japan or Rhine-Hesse clone) has high worldwide prevalence (11). It was the most prevalent MRSA type in the 2000s in Hungary, until its replacement by ST22-IV MRSA (75). ST5-II is a classical HA-MRSA strain, described more frequently in older patients than in children (109). ST5 and ST225 types are indistinguishable by PFGE, and both were prevalent in Germany (110).

A representative of our PFGE type C (Figure 9a) isolates belonged to ST1-I, a non-epidemic clone, which was found in a low number of patients, for example, in Croatia and in Italy (111, 112).

Similarly to other studies, we have found high diversity among MSSA isolates, no predominant clone was recognised (Figure 9b) (74, 113).

4.2.4.1. Role of SCCmec type in resistance, virulence and mortality of infected patients in MRSA BSI

From the MRSA isolates in our study, SSC*mec* I and II isolates were associated with the highest resistance rates to antibiotics, whereas SCC*mec* IV isolates were sensitive to almost all antibiotics except for ciprofloxacin and macrolides (Table 7). High replication rate in spite of high ciprofloxacin resistance may be the driving factor behind the ongoing expansion of ST22-IV clone (75), as was described in chapter 4.1. as well.

According to the literature, SCC*mec* types I-II-III are more likely to exhibit high glycopeptide MIC values and vancomycin hetero-resistance, than SCC*mec* IV isolates (114, 115). This is in concordance with our findings. In our study, high vancomycin and teicoplanin values (MIC=2 mg/L) were approximately 5 times more frequent in SCC*mec* I and 2 times more frequent in SCC*mec* II compared to SCC*mec* IV isolates (Table 7).

SCC*mec* II isolates carried the highest number of virulence genes, 7 on average, while median number of virulence determinant in SCC*mec* II and IV isolates was 5.5 and 6, respectively (Table 8). All of our *pvl* positive MRSA isolates belonged to SCC*mec* type I or II. Earlier studies associated PVL positivity with CA-MRSA, but later HA-MRSA strains carrying *pvl* genes were also described. The diffusion of *pvl* genes into various MRSA lineages is mediated via PVL bacteriophages and rises concerns about the possibility of emergence of multiresistant and highly virulent HA-MRSA strains (116).

Although SCC*mec* type I isolates were the most resistant to antibiotics and SCC*mec* type II had the most virulence genes, interestingly SCC*mec* type IV isolates had comparable associated mortality rates – in fact, mortality was the highest in this group (42.2% in SCC*mec* IV vs 28.6% and 36.1% in SCC*mec* types I and II, respectively) (Table 9), although the difference was not significant statistically. Baseline CCI of patients did not differ significantly. SCC*mec* IV was the most prevalent type among our BSI samples, and has successfully replaced the previously widespread epidemic clones in Hungary and in many other parts of the world. The successful pandemic spread of the ST22-IV clone and the associated high mortality remains an interesting topic for research. Faster replication rate of the ST22-IV clone compared to other international clones may play an important role in this success (75).

As it was described by Recker et al., bacterial phenotype and genotype are highly predictive of infection outcome, and have more influence on mortality than other factors, such as patient age, gender or comorbidities (113). This underlines the importance of up-to-date knowledge on the clonal types circulating at a given location.

5. CONCLUSIONS

Fluoroquinolone antibiotics play a crucial role in the selection of international clones of *S. aureus*. Strains that are able to maintain high replication rate in parallel to developing high level of fluoroquinolone resistance have a great advantage over other clones in hospital settings, where fluoroquinolone antibiotics are frequently used. This observation may provide an explanation for the selection of the globally successful major clones of *S. aureus*.

CA-MRSA strains, which suffer high fitness lost upon acquisition of fluoroquinolone resistance will not be able to take ground in healthcare setting. In our study, we demonstrated this overwhelming decrease in fitness by inducing fluoroquinolone resistance in ST8-IV, ST80-IV and ST30-IV CA-MRSA strains. Upon acquisition of mutations in the QRD regions of their appropriate genes, these strains suffered great loss of vitality, proving that they are capable of developing resistance, however, in their resistant form, they cannot compete with the higher replication rates of successful HA-MRSA clones.

Moreover, differences in fitness cost related to fluoroquinolone resistance shed light on the phenomenon of clonal replacement. According to our data, strains of ST5-II and particularly ST22-IV clones are able to sustain a high replication speed while exhibiting high fluoroquinolone MIC values. On the contrary, strains of ST239-III and ST228-I clones are less fit and replicate considerably slower. These data suggests that a dominant clone in an area may get replaced by another emerging one, if the new clone is able to withstand the effect of antibiotics in the setting, and is able to better maintain the speed of replication despite mutation in QRDR. Such new clones are able to outcompete and surpass previously dominant clones.

Most fluoroquinolone resistant clones, however, lose their superiority in fluoroquinolone-free environment, as most of them cannot compete with the replication speed of fluoroquinolone susceptible strains. ST22-IV MRSA, an internationally successful major clone is a remarkable exception, as it has comparable replication speed to fluoroquinolone susceptible MRSA clones (75).

Because of the decisive role of fluoroquinolone antibiotics in the selection and rise of successful *S. aureus* clones, more judicious use of these antibiotics could help in the regress of the major clones which is described to be associated with decline in the incidence of HA-MRSA.

Our study on the *S. aureus* isolates causing BSI over a 6-year time period revealed differences in antibiotic resistance, virulence factors and associated mortality in infections caused by different types of *S. aureus*. Outcome of the infection was much worse in patients with MRSA BSI. Mortality rates were also higher in females, older patients, and in patients who had BSI caused by SCC*mec* IV isolates, and by isolates with high teicoplanin MIC. MRSA isolates had much higher resistance rates, MDR was also more frequent among them. MRSA strains also carried more virulence genes, and different types compared to MSSA. We have found low prevalence of PVL and superantigens. Number and type of carried virulence factors was not associated with increased mortality rates.

Among MRSA isolates, we have found that similarly to other parts of the world, the vast majority of the strains belong to ST22-IV clone. Isolates of ST5-II, ST225-II and ST1-I were also found among our samples. MRSA isolates of different clones had different characteristics. SCC*mec* I and II isolates had high rates of antibiotic resistance in general, and particularly high glycopeptide MIC values. SCC*mec* II isolates had the highest number of virulence genes. Interestingly, PVL was found exclusively in SCC*mec* I and II isolates. Surprisingly, despite these advantages of SCC*mec* I and II isolates, mortality of BSI caused by SCC*mec* IV isolates was higher than that of SCC*mec* I and II MRSA infections. SCC*mec* IV isolates maintained their dominance in our samples during the six years of the study.

The novel observations concluded based on this thesis are the following:

- Isolates of CA-MRSA clones suffer greater loss of fitness upon developing fluoroquinolone resistance and this renders them unable to compete with the successful major HA-MRSA clones in healthcare settings.
- Fluoroquinolones have a crucial role in selection of HA-MRSA clones.

- HA-MRSA isolates of different clones also differ in their speed of replication. ST5-II and particularly ST22-IV strains are able to maintain high replication rate in spite of their fluoroquinolone resistance. This observation could provide an explanation for the expansion of these clones over the past ten years.
- In our study, we described and compared the antibiotic resistance and virulence patterns of MRSA and MSSA BSI isolates for the first time in Hungary. We have found higher antibiotic resistance rates, higher MDR rate and also higher number of carried virulence determinants in MRSA compared to MSSA. Mortality of MRSA BSI was significantly higher than that of MSSA BSI, as well.
- We provided insight into the current clonal composition of BSI *S. aureus* isolates in Hungary. We identified ST22-IV as the dominant clone in the recent years.
- We compared isolates of different MRSA clones, and found that isolates of SCC*mec* I and II clones have the highest antibiotic resistance rates, SCC*mec* II isolates carry the most virulence factors, nevertheless, SCC*mec* IV MRSA infections are associated with the highest mortality rates.
- Our results suggest that the outcome of the infection is determined by the antibiotic resistance and genotype of the bacterium; and patient related factors rather than the virulence determinants of the pathogen.
- Based on our study regarding the impact of fluoroquinolone resistance on the fitness cost of various MRSA clones, we suggest that the success of ST22-IV clone, and its role in the replacement of the previously dominant clones by this type, moreover, the sustained dominance of this clone during our study and the high mortality rate of patients with BSI caused by the ST22 clone is likely the result of greater fitness and higher replication rate of this clone compared to other MRSA clones in our region.
- Selective pressure of fluoroquinolones could be reduced by the more judicious use of these antibiotics, which could result in reduction of the major MRSA clones and in the overall rate of MRSA.

6. SUMMARY

S. aureus is a major human pathogen, frequently causing life-threatening infections in hospitals as well as in the community. *S. aureus* is frequently resistant to antibiotics of different classes. MRSA expresses resistance not only to β-lactams, but to antibiotics of other classes, too.

Population structure of MRSA is highly clonal, a few successful clones dominate in a given area and time period. Clones of MRSA may differ from each other in terms of level of resistance, virulence, and in types and outcome of the caused infections. Different clones compete with each other in a niche.

In this study we examined the effect of fluoroquinolone resistance on the fitness (the capacity to replicate) of various MRSA clones. We have found that isolates of CA-MRSA clones can be induced to develop resistance to fluoroquinolones. However, during this process they suffer that great loss of vitality disabling them to disseminate into healthcare settings and compete with major HA-MRSA clones. This finding suggests that the use of fluoroquinolones has a crucial role in the selection of successful HA-MRSA clones. Isolates of ST22-IV clone are especially remarkable in their speed of replication and level of fluoroquinolone resistance. This could provide an explanation for the success of this globally disseminated clone.

We also studied the composition of *S. aureus* strains currently causing BSI in Hungary. MRSA were more resistant to antibiotics and carried more virulence genes compared to MSSA. They were also associated with much higher mortality rates. Outcome of the infection was worse in females, in older patients and in those infected with SCC*mec* IV isolates or with isolates of high teicoplanin MIC. Isolates of ST22-IV clone dominated throughout the six years of our study. We described considerable differences between MRSA isolates of different clones. SCC*mec* I and II MRSA isolates were the most resistant to antibiotics and exhibited high glycopeptide MIC values. Isolates of SCC*mec* type II carried especially high number of virulence factor genes. However, SCC*mec* type IV isolates were associated with the highest rates of mortality. Dominance of this clone and high rate of mortality of patients infected with it may be the result of the higher level of fitness and greater replication rate of this clone compared to other MRSA types.

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8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

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