TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
HEALTH SCIENCES INSTITUTE

INVESTIGATION OF STAPHYLOCOCCAL α-HEMOLYSIN AND EXFOLIATIVE TOXIN A IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL

TCHAMOU MALRAUX FLEURY POTINDJI

MASTER THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY DEPARTMENT

ADVISOR
ASSOC. PROF. BUKET BADDAL

NICOSIA, 2021

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The Directorate of Health Sciences Institute,

This study has been accepted by the Thesis Committee in Medical Microbiology and Clinical Microbiology Program as a Master of Science Thesis.

Thesis committee:

Chair:

Supervisor: Assoc. Prof. Buket BADDAL, Near East University

Members:

Approval:

According to the relevant articles of the Near East University Postgraduate study-Education and Examination Regulations, this thesis has been approved by the abovementioned members of the thesis committee and the decision of the Board of

Directors of the institute.

Professor K. Hüsnü Can BAŞER, PhD

Director of Graduate School of Health Sciences

DECLARATION

I hereby declare that the work in this thesis entitled "**INVESTIGATION OF STAPHYLOCOCCAL α-HEMOLYSIN AND EXFOLIATIVE TOXIN AIN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL**" is the product of my own research efforts undertaken under the supervision of Assoc. Prof. Buket Baddal. No part of this thesis was previously presented for another degree or diploma in any university elsewhere, and all information in this document has been obtained and presented in accordance with academic ethical conduct and rules. All materials and results that are not original to this work have been duly acknowledged, fully cited and referenced.

Name, Last Name:

Signature:

Date:

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ABSTRACT

Tchamou Malraux Fleury Potindji. Investigation of Staphylococcal α-Hemolysin and Exfoliative Toxin A in Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates at Near East University Hospital. Near East University, Institute of Health Sciences, Medical Microbiology and Clinical Microbiology Program, M.Sc. Thesis, Nicosia, 2021

Staphylococcus aureus is a leading cause of human bacterial infection worldwide. As a member of human microbiota, individuals can be asymptomatically colonized and spread *S. aureus* infections to susceptible individuals. Infections caused by *S. aureus* can range from mild to severe conditions. The emergence and rapid spread of antibiotic resistant strains constitute a challenge that need to be timely addressed. Over the years, *S. aureus* has become resistant to β-lactam antibiotics, with a special regard to community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains which have appeared increasingly involved in nosocomial infections. The extent of an infection depends on the virulence factor associated in the pathogenesis. Indeed, *S. aureus* produces a large set of virulence factors involved in pathogenesis, including: Panton-Valentine leukocidin (PVL), super antigens, exfoliative toxins, phenol-soluble modulins and α-toxin. The aim of this thesis project was to investigate the presence of α-toxin and exfoliative toxin A genes in MRSA clinical isolates at Near East University Hospital. Seventy-six MRSA isolates were identified using coagulase tube test, cefoxitin disc diffusion test and *nuc* gene amplification by polymerase chain reaction (PCR). α-toxin gene and exfoliative toxin A gene were detected in 97.36% and 2.63 % of the isolates respectively. This study demonstrates the first report to investigate hemolytic and epidermolytic toxin carriage in MRSA in Northern Cyprus and has laid the path to further comprehensive studies.

Keywords: *Staphylococcus aureus*, MRSA, virulence factors, α-toxin, exfoliative toxin A

ÖZET

Tchamou Malraux Fleury Potindji. Yakın Doğu Üniversitesi Hastanesi'nde Metisilin Dirençli *Staphylococcus aureus* Klinik İzolatlarında Stafilokokal α-Hemolizin ve Eksfoliyatif Toksin A'nın Araştırılması. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Programı, Yüksek Lisans Tezi, Lefkoşa, 2021

Staphylococcus aureus, dünya çapında bakteriyel enfeksiyonların önde gelen nedenlerinden biridir. Asemptomatik bireylerin *S. aureus* ile kolonize olabilmesi, duyarlı kişilerde *S. aureus* enfeksiyonu yayılım riskini artırmaktadır. *S. aureus'*un neden olduğu enfeksiyonlar, hafif veya şiddetli olarak seyredebilmektedir. Antibiyotiğe dirençli suşların ortaya çıkması ve hızlı yayılımı, insan sağlığı için bir tehdit oluşturmaktadır. Son yıllarda *S. aureus* suşlarında görülen β-laktam direnci ile toplum-kökenli metilisin-dirençli *S. aureus* (MRSA) nozokomiyal enfeksiyonlarda sıklıkla görülmektedir. Enfeksiyonun seyri, patogenezle ilişkili virülans faktörüne bağlıdır. *S. aureus,* Panton-Valentine lökosidin (PVL), süper antijenler, eksfolyatif toksinler, fenol-çözünür modülinler ve α-toksin dahil olmak üzere patogenezde rol oynayan geniş spektrumlu virülans faktörlerine sahiptir. Bu projenin amacı, Yakın Doğu Üniversitesi Hastanesi'nde izole edilen klinik MRSA suşlarında α-toksin ve eksfolyatif toksin A gen varlığının incelenmesidir. Tüp koagülaz testi, sefoksitin disk difüzyon testi ve polimeraz zincir reaksiyonu (PZR) *nuc* gen amplifikasyonu kullanılarak 76 MRSA izolatı konfirme edilmiştir. İzolatlarda α-toksin ve eksfolyatif toksin A genleri suşların sırasıyla %97.36 ve %2.63'ünde tespit edilmiştir. Bu çalışma, Kuzey Kıbrıs'ta MRSA izolatlarında hemolitik ve epidermolitik toksin varlığını araştıran ilk verileri raporlamakla birlikte, ileriki kapsamlı çalışmalara ışık tutmaktadır.

Anahtar kelimeler: *Staphylococcus aureus,* MRSA, virülans faktörü, α-toksin, eksfolyatif toksin A

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LIST OF ABBREVIATIONS

MSCRAMM: microbial surface components recognizing adhesive matrix molecules

hla: α-toxin gene

exfA*:* exfoliative toxin A gene

nuc: thermonuclease gene

PBP: penicillin binding protein

SCC**:** staphylococcal cassette chromosome

MSSA: methicillin sensitive *Staphylococcus aureus*

MRSA: methicillin resistant *Staphylococcus aureus*

CA-MRSA: community-associated methicillin resistant *S. aureus*

HA-MRSA: healthcare-acquired methicillin resistant *S. aureus*

LA-MRSA: livestock-associated methicillin resistant *S. aureus*

ECDC: European Center for Disease Prevention and Control

EUCAST: European Committee on Antimicrobial Susceptibility Testing

CoPS: coagulase positive staphylococci

CoNS: coagulase negative staphylococci

IS: insertion sequence

Tn: transposon

CP: capsular polysaccharide

LTA: lipoteichoic acid

WTA: wall teichoic acid

PFT: pore forming toxin

ADAM-10: a disintegrin and metalloprotease 10

PLEKHA7: Pleckstrin Homology Domain Containing A7

PVL: Panton-Valentine leukocidin

SAg: super antigen

SAE: *S. aureus* enterotoxins

ET: exfoliative toxin

ETA: exfoliative toxin A

TSS: toxic shock syndrome

TSST: toxic shock syndrome toxin

SSSS: staphylococcal scalded skin syndrome

SSSI: skin and skin structure infection

TCS: two component system

agr: accessory gene regulator

ICU: intensive care unit

NLR: node-like receptor

PSM: phenol-soluble modulin

MDR: multidrug resistant

TRNC: Turkish Republic of Northern Cyprus

WHO: World Health Organization

SECTION ONE: INTRODUCTION

1. Aims and Scope

The impact of antibiotic resistance traits and virulence factors of pathogenic bacteria, on disease pathogenesis and clinical outcome is a well-documented aspect of clinical microbiology. These factors are major concern to global public health, and are needed to be promptly and comprehensively addressed.

Sir Alexander Ogston introduced the name *Staphylococcus* in 1880 to describe a group of bacteria causing suppurative infections, due to their tendency to form grape-like clusters. In 1884, Rosenbach isolated *Staphylococcus aureus* (*S. aureus*) (golden colonies on culture media) and *Staphylococcus albus* (white colonies) (Licitra, 2013). Staphylococci are widespread in the environment, though, *S. aureus* is mainly found as a commensal on human skin and mucous membranes, with approximately 30% of human individuals being asymptomatic and persistent carriers (Sakr et al., 2018). Indeed, *S. aureus* carriage has been well correlated with infection rates in human, particularly in individuals with risk factors such as: underlying disease, active smoking and immunodeficiency) (Akhtar Danesh et al., 2020; Sakr et al., 2018).

S. aureus, as a prominent human pathogen, is a leading cause of bacterial infection worldwide. *S. aureus* is capable of infecting a variety of host species (Matuszewska et al., 2020), causing a wide range of human diseases: (i) superficial infections such as skin and soft tissues infections (SSTIs), from benign to life threating conditions (impetigo, cellulitis, surgical sites infections, cutaneous abscesses, purulent cellulitis) (MacMorran et al., 2017); atopic dermatitis (Geoghegan et al., 2018; Hepburn et al., 2017); (ii) systemic and life threatening infections such as blood stream infection (Kourtis et al., 2019), intravascular catheter infections (Austin et al., 2016; Sato et al., 2017) ; infective endocarditis, pleura-pulmonary infections; (iii) toxin-induced conditions such as food poisoning, toxic shock syndrome (Tong et al., 2015), and other staphylococcal clinical syndromes including urinary tract infections, epidermal abscesses and meningitis. Such versatility emerges from the pathogen's ability to secrete diverse host damaging virulence

factors. Among the multiple virulence factors produced by *S. aureus*, it is possible to enumerate, cell surface antigens such as clumping factor, protein A, Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), that helps attachment to host tissue (Foster, 2019); secretion of enzymes, namely, proteases, nucleases, hyaluronidase, coagulase, staphylokinase can cause tissue destruction and thereby, helps bacterial penetration (Tam & Torres, 2019). On the other hand, toxins are proteins secreted into the extracellular matrix; they for example, as staphylococcal enterotoxins (SETs), may display a potent gastrointestinal pathogenicity and cause intoxication (Fisher et al., 2018); or as exfoliative toxins, disrupt epidermal layers causing staphylococcal-scalded skin syndrome, a disease predominantly affecting infants; or as super-antigens, interfere with receptor function (Kim, 2019). More importantly, cytotoxic activity seen in pore forming toxins, particularly α-toxin, is of great interest. α-toxin, also known as α-hemolysin or *hla* is a protein that injures the human cells through its homo-heptamer pore forming capabilities (von Hoven et al., 2019).

The emergence of antimicrobial resistance traits, and more particularly, resistance to methicillin have become widespread and has led to resistant strains. Resistance to methicillin and other β-lactams, such as oxacillin, penicillin, cephalosporin's results from the acquisition of *mecA* gene which resides on a staphylococcal chromosome cassette (*SCCmec*) (Foster & Geoghegan, 2015). Penicillin binding proteins (PBP's) are enzymes that play key role in biosynthesis of major components of bacterial cell wall (Shalaby et al., 2020). In the presence of β-lactams, the native PBP's are inactivated, thus, cell wall major components synthesis is blocked; such process is lethal for the bacterial cell. The *mecA* gene expression produces penicillin-binding protein 2a or 2' (PBP2a or PBP2'), a modified penicillin-binding protein which is not inactivated by β-lactams (due to lower affinity); PBP2a is able to take over the cell wall synthesis in place of native PBP's, giving rise to methicillin resistant *Staphylococcus aureus* (MRSA) emergence (Kirmusaolu, 2017). General misuse of antibiotics is known to have led to the selection of hospital-acquired (HA) MRSA as well as community-acquired (CA) MRSA.

Given the strong association between pathogenicity level (including virulence factors and resistance traits) and severity of clinical outcome evidenced by numerous studies (Bennett & Thomsen, 2020; Joo et al., 2019; Mao et al., 2019); and the emergence and spread of both CA-MRSA (Kateete et al., 2019) and HA-MRSA (Lee et al., 2018),

having greater insights into MRSA is therefore invaluable for infection control in local hospitals.

According to the European Center for Disease Prevention and Control (ECDC) prevalence survey in 2011-2012, *S. aureus* was the second most common cause of healthcare associated infections (12.3%), with 41.2% reported with resistance to methicillin (ecdc, 2013). In 2015, in Cyprus, 43.4% of *S. aureus* invasive isolates were reported to be MRSA (European Centre for Disease Prevention and Control (ECDC), 2016).

A closer look to the literature reveals a number of areas in MRSA infection, epidemiology and pathogenesis with limited datasets in Turkish Republic of Northern Cyprus (TRNC). This may raise concern about the challenging issue of *S. aureus* infection management in the country. In response to the need, the present study aims to investigate the presence of staphylococcal α-toxin gene (*hla*) and exfoliative toxin A gene (*exfa*) genes in MRSA isolates from patients attending Near East University Hospital.

2. General Information

2.1. The genus Staphylococcus

Members of the genus *Staphylococcus* are Gram-positive cocci, 0.5-1.5 μmin diameter, that occur single, in pairs, tetrads or irregular grape-like clusters **(**Figure 1**)**. They are non-motile, non-spore forming, non-encapsulated (or with limited capsule formation). They are halotolerant (growth in the presence of 10% sodium chloride) and most of the species are facultative anaerobe (except *S. saccharolyticus* and *S. aureus* subsp *anaerobius)*. Most species exhibit catalase activity distinguishing them from the genus *Streptococcus* and are oxidase negative (Gherardi et al., 2018). To date, overall 45 species and 24 subspecies have been described.

Coagulase positive staphylococci (CoPS), predominantly represented by *S. aureus*, possess coagulase; conversely those strains which do not possess coagulase enzyme are termed coagulase negative staphylococci (CoNS).

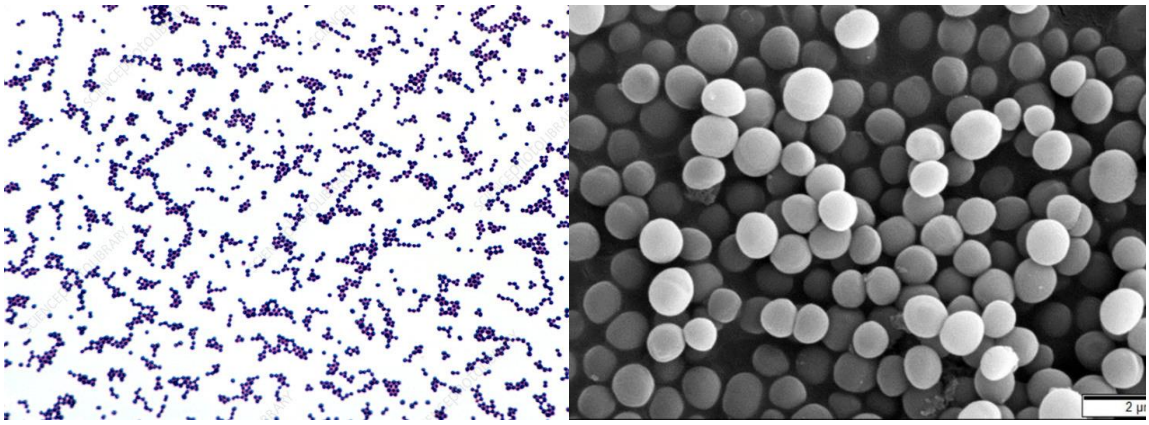


Figure 1: *S. aureus* micrographs by Gram-staining and electron microscopy imaging (Murtey & Ramasamy, 2016)

2.2. *Staphylococcus aureus*

S. aureus is a major pathogen with an increasing importance due to the emergence of antibiotic resistance (Foster, 2017). The bacterium is a human commensal and may act as a pathogen when it gains access to a normally sterile site due to traumatic inoculation (Dayan et al., 2016).

2.3 Laboratory identification

Clinical isolates of staphylococci species can be identified using Gram staining, colony morphology, biochemical tests including catalase, coagulase production, antibiotic resistance traits, hemolysins, carbohydrates degradation ability such as mannitol fermentation.

S. aureus colonies appear round with regular edges and typical golden appearance on blood agar. They are frequently surrounded by a zone of clear β hemolysis. Although a variety of biochemical test can be used to confirm *S. aureus* such as nuclease, catalase, clumping factor tests, coagulase test remain the gold standard (Aryee & Edgeworth, 2016). *S. aureus* is both catalase and coagulase positive. Regarding antimicrobial

susceptibility testing, the Kirby-Bauer disk diffusion test is the most widely used approach as it allows to test bacterial susceptibility to various antibiotics simultaneously. Cefoxitin disk diffusion method is used to rule out the phenotypic presentation of resistance to methicillin and distinguish MRSA from methicillin sensitive *S. aureus* (Kriegeskorte et al., 2017). Of note, *S. aureus* is currently resistant to most of the antibiotics used (Foster, 2017). Moreover, rapid development of molecular identification systems, renders detection of *S. aureus* timely and comparably more accurate.

2.4 The chromosome

Whole genome sequencing of *S. aureus* chromosome has yielded a comprehensive overview of genomic arrangement. The genome (2.820 Mb to 2.903 Mb in size), is composed of a single circular chromosome, with a collection of extrachromosomal accessory genes elements.

2.4.1 The core genome

S. aureus core genome is approximately 2.3 Mb in size and is responsible for common functions such as metabolism control and other maintenance ‘’housekeeping’’ functions; as well as many virulence factor genes. This part of the genome is highly conserved among the different strains and represent approximately 75% of the whole genome (Chua et al., 2013).

2.4.2 Mobile elements of the genome

Accounting for around 25%, mobile genetic elements consist of diverse elements such as: plasmids, pathogenicity islands, transposons, chromosomal cassettes (Lindsay & Holden, 2004). They are DNA fragments encoding for numerous proven virulence and resistance factors. They are termed as *mobile genetic elements*, due to their high inter-

strain transfer frequency, and are from a functional point of view, not essential (Alibayov et al., 2014).

2.4.2.1 Insertion sequences and transposons

Insertion sequences (*ISs*) and transposons (*Tns*) are transposable genetic elements. They carry genes responsible for the expression of proteins involved in transposition activity. Only transposons carry accessory virulence genes, such as antibiotic resistance genes. Although *ISs* do not carry any resistance gene, their presence is very important for the stabilization of some virulence genes (Foster & Geoghegan, 2015). Termed as transposable elements, they are partly responsible for the genome plasticity.

2.4.2.2 Plasmids

S. aureus plasmids have been grouped into three classes, class I, II and III according to their relative size and the genes they possess. These plasmids carry a various collection of genes encoding resistance to antibiotics and heavy metals as well as resistance to detergents. Class III plasmids are mostly conjugative plasmids (Foster & Geoghegan, 2015). For example, *blaZ* gene, responsible for resistance to penicillin is harbored by a transposable element that can be found either in the core chromosome, or, as part of a plasmid which additionally encode for heavy metal resistance (Vestergaard et al., 2019).

2.4.2.3 Staphylococcal chromosome cassette: *SCC*

SCC elements are relatively large mobile genetic elements that can carry several sets of virulence genes. The most widely acknowledged *SCC* element is the *SCCmec* cassette that carry the *mec* complex which contains the *mecA* gene. This gene encodes a unique penicillin-binding protein PBP2a, responsible for low affinity to β-lactam

antibiotics, as well as other functional genes (Alibayov et al., 2014). All *S. aureus* strains resistant to methicillin carry a *SCCmec* cassette, and to date, up to 13 different types of *SCCmec* have been identified (Baig et al., 2018).

All *SCCmec* elements share the same molecular structure that consist of : (i) the ***mec* complex**, which carries the *mecA* gene as well as its regulatory genes namely *mec*I (repressor) and *mec*RI (trans-membrane β-lactam sensing signal transducer) and insertion sequences; the ***ccr* complex**, which mediates integration into and excision of *SCCmec* at specific sites of the bacterial genome; and, the **J regions**, stands for 'joining' regions, are regions bordering the *mec* and *ccr* complexes sites **(Figure 2)** (Carretto et al., 2018)**.**

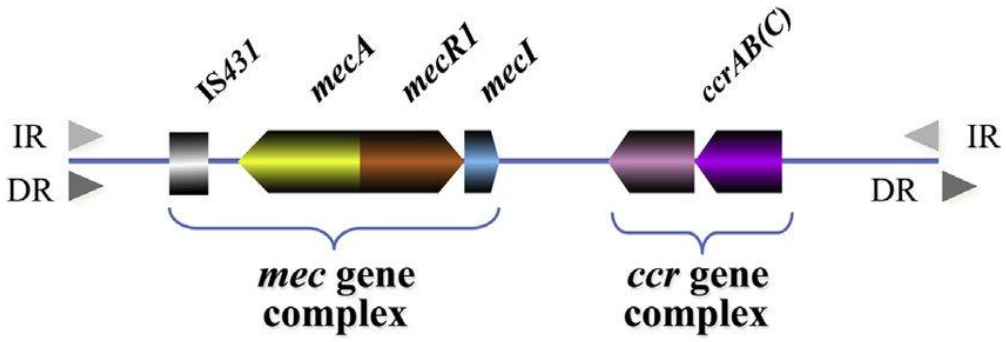


Figure 2: Schematic structure of the *SCCmecA*. Presentation of the *mecA* gene complex along with its regulatory components; the ccr gene complex and the J regions at the extremities. *SCCmec* is bracketed by direct repeats (DRs) that contain integration site sequence (ISS) recognized by cassette chromosome recombinase (CCR). A pair of inverted repeats (IRs) (Hiramatsu et al., 2014)

Currently, other genes coding for resistance to methicillin in S. aureus have been described; *mecC* (Dupieux et al., 2017; Kriegeskorte et al., 2017), *mecD* (Schwendener et al., 2017) and *mecB* (Lakhundi & Zhang, 2018). Other known mobile genetic elements of *S. aureus* are bacteriophages, genomic and pathogenicity islands (Lindsay & Holden, 2004).

2.5. *S. aureus* cell surface virulence factors

S. aureus cell wall organization shows similar architecture to that of other gram-positive bacteria. The first structure to be described is the surrounding polysaccharide capsule that act as a distinct virulence factor able to trigger host response **(Figure 3)** (B. Liu et al., 2017). Major capsular polysaccharides (CP), CP5 and CP8, are demonstrated to impede opsonization and phagocytic killing by polymorphonuclear neutrophils which is considered immune system's primary mechanism of pathogen clearance and allow the bacteria to persist. It has been shown that high capsule expression in a certain strain is consistent with high resistance to *in vitro* opsonophagocytic killing (Nanra et al., 2013), and correlates with higher virulence in encapsulated strains in comparison to capsule-defective mutants (Watts et al., 2005).

Teichoic acids are located within the cell wall. They are either incorporated into the plasma membrane as lipoteichoic acids (LTAs), or to the cell wall as wall teichoic acids (WTAs). Several host receptors interact with teichoic acids, yielding functional effects as various as adhesion, activation of dendritic cells and Langerhans cells (epidermidis and mucosal tissue resident immune cell, thus playing an important role in *S. aureus*-related skin pathology) and detection by serum components (Kang et al., 2016; van Dalen et al., 2020). Moreover, they mediate interaction with biomaterials and contribute to biofilm formation, control protein machinery of the cell or serve as phage receptors (Xia et al., 2010).

Surface associated proteins, also termed as cell wall anchored proteins, are covalently linked to the peptidoglycan (Becker, 2018). It is a super family that perform functions ranging from adhesion and invasion of host cell, to immune evasion. The MSCRAMM is the most prevalent family, it includes cell surface proteins such as clumping factor (Clf) A and B, fibronectin-binding protein (FnBP) A and B involved in biofilm formation, biofilm associated protein (Bap), which interact with host extracellular matrix molecules and promote attachment to tissue as well as evasion from immune system (Foster, 2019).

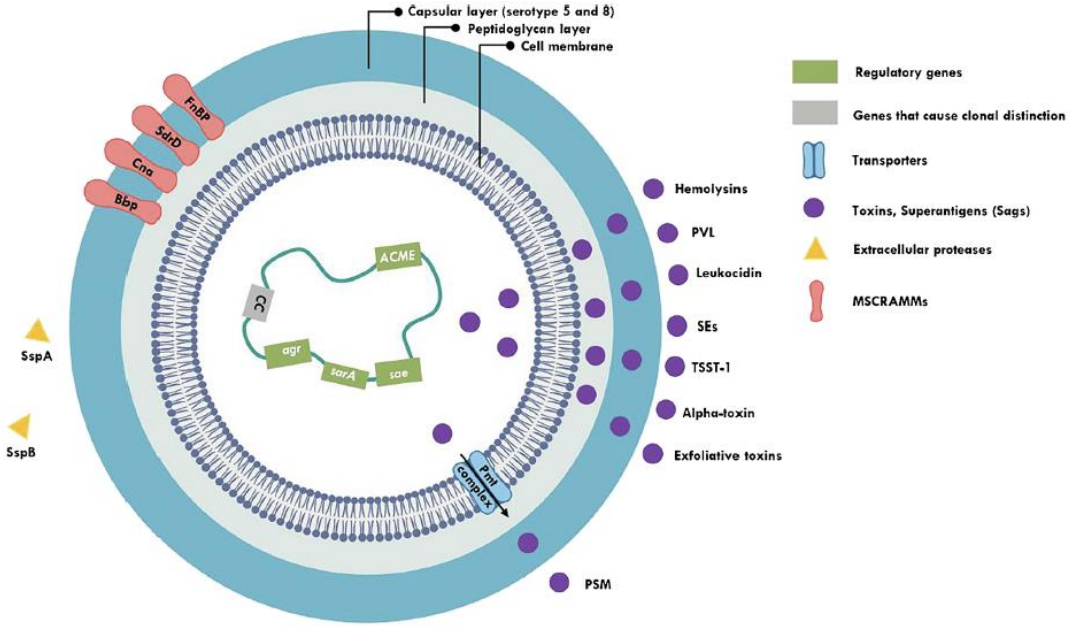


Figure 3: Schematic diagram of *S. aureus* virulence determinants (Shettigar & Murali, 2020)

2.6. Secreted virulence factors

S. aureus produces several sets of virulent exoproteins which reflects the ability of the pathogen to cause a variety of diseases **(Figure 4)**. Staphylococcal toxins can be classified as follows: (i) the cytolysins, that exhibit cytolytic activity; (ii) exotoxins with super-antigen (SAg) activity and (iii) specific enzymes. The first two groups have been extensively studied and correlated with disease.

2.6.1. α-hemolysin

Cytolytic toxins is the group of membrane damaging toxins that exhibit cytolytic effect toward cells. First, β-barrel pore forming toxins (PFTs) form β-barrel pores in the plasma membrane of the target cells, inducing cell death. *S. aureus* is known to produce such toxins that target different cell surface receptors and exhibit a very wide range of cell and species specificities (Tam & Torres, 2019).

One of the most studied member of this subgroup is *α-hemolysin*: the prototypic β-barrel PFT; its functions have been described as direct cell toxicity on many cell types, immune-modulation through initiation of pro-inflammatory signaling and induction of cytokines secretion (Berube & Wardenburg, 2013). In 2010, Wilke et al. have identified the cell surface receptor A disintegrin and metalloprotease 10 (ADAM-10) as a membrane requirement for *α-hemolysin* to exert its action, thus required for the initiation of cascade signaling by which the toxin monomer polymerizes into a transmembrane cytolytic pore (Wilke & Wardenburg, 2010). *α-hemolysin* is of high relevance in a variety of staphylococcal infection models in animal; its signaling effect intensity have been correlated with factors such as: (i) the cell type, (ii) the relative concentration of the toxin, (iii) the level of ADAM-10 expressed on the cell (Berube & Wardenburg, 2013; Seilie & Bubeck Wardenburg, 2017; Tam & Torres, 2019). More recently, PLEKHA7 (plekstrin-homology domain containing protein 7), a cellular adherens junction component, has additionally been reported to play a critical role in *α-hemolysin* toxicity, and susceptibility to epithelial infections in an animal model (Popov et al., 2015).

2.6.2. Bicomponent PFTs

Another major cytotoxin group is the bicomponent pore forming toxin group. It is a group of toxins with large effect toward monocytes, macrophages, neutrophils and erythrocytes (Oliveira et al., 2018). The first member of this group to be purified is Panton-Valentine leucocidin (PVL), a bi-component leukocidin PFT which consists of two subunits: LukF-PV and LukS-PV; PVL exhibit a prevalent specificity for leucocytes (Tam & Torres, 2019), and is mostly associated with CA-MRSA (Bennett & Thomsen, 2020). The pore formation is the result of interactions between the subunits F, S and the host cell surface proteins. The formed pore exhibits the typical three domain structure (Cap, Rim and Stem domain). PVL genes are carried within lysogenic phage genes (Nawrotek et al., 2018); and at least eight different lysogenic phages are known to carry PVL genes.

γ-hemolysins are Hla AB and Hla CB. They both share the same F subunit (Hla B), but differ in their S subunit (Hla A and Hla C) (Oliveira et al., 2018). They are

cytotoxic towards red blood cells and the genes responsible for their expression is located in the core genome and present in 99% of *S. aureus* strains (Tam & Torres, 2019).

Other leukocidins, Luk ED and Luk AB are cytotoxic for leukocytes. The first is highly conserved among 70% of strains and the latter is present in the genome of 99% of *S. aureus* strains (Tam & Torres, 2019).

2.6.3. Phenol soluble modulins

Phenol soluble modulins are amphipathic peptide toxins that are specific to staphylococci (Tam & Torres, 2019). They are genome encoded toxins and are divided into two subfamilies: (i) PSMα (which include δ-hemolysin) are short amino-acid chain (20-25 amino-acids) toxins; (ii) PSMβ with longer amino-acids chain (40-45 amino-acids). They are primarily regulated by the *agr* system in an RNA III-independent manner and may function as toxins, assist biofilm formation (Oliveira et al., 2018) and act as pro-inflammatory inducers (Geoghegan et al., 2018).

2.6.4. Superantigens

S. aureus superantigens (SAgs) are potent T-cell mitogens and can induce their non-specific activation and proliferation. They are divided into three different subclasses: (i) staphylococcal enterotoxins (SAEs), agents of food-borne diseases; (ii) staphylococcal enterotoxin-like toxins and (iii) toxic shock syndrome toxin (TSST), agent of toxic shock syndrome (TSS) (Tam & Torres, 2019). The hallmark of their activity is due to their ability to induce nonspecific activation of immune T cell, resulting in massive clonal expansion, overproduction of cytokines (cytokine storm), and exacerbated inflammation. Over 26 SAgs have been identified to date (Bennett & Thomsen, 2020). TSS is a severe striking condition defined by fever, rash/desquamation and in ultimate case, multi organ failure. Besides, staphylococcal protein A (spa) is the only B cell SAg described, with an Fc portion of immunoglobulins binding activity, thus preventing opsonophagocytic killing (Bennett & Thomsen, 2020).

2.6.5. Exfoliative toxins

Exfoliative toxins (ETs) is the group of toxins showing epidermolytic activity by cleaving cell-to-cell focal adhesions in epidermis (Oliveira et al., 2018). The characteristic manifestation is the skin exfoliation seen in patients suffering from staphylococcal scalded skin syndrome. There are four ETs present in *S. aureus*: ETA, ETB, ETC and ETD; each form is antigenically distinct from the others (Tam & Torres, 2019).

2.6.6. Exoenzymes and specific proteins

S. aureus secrete other enzymes with specific activity: (i) staphylococcal coagulase, which promotes survival and escape from immune system; (ii) spreading factors such as staphylokinase and hyaluronidase which cleaves hyaluronic acid, a major host extracellular matrix component, nucleases, lipases and proteases(Tam & Torres, 2019). Evasion of innate immune system is also mediated through secreted and cell surface components such as: (i) staphylococcal complement inhibitor (SCIN), a C3 convertase inhibitor; (ii) chemotaxis inhibitory protein of *S. aureus*, which inhibit chemotactic migration of neutrophils; (iii) extracellular adherence protein (Eap) inhibits diapedesis;(iv) superoxide dismutase, which enable phagosome survival in the case of bacterium being engulfed by neutrophils (Foster & Geoghegan, 2015).

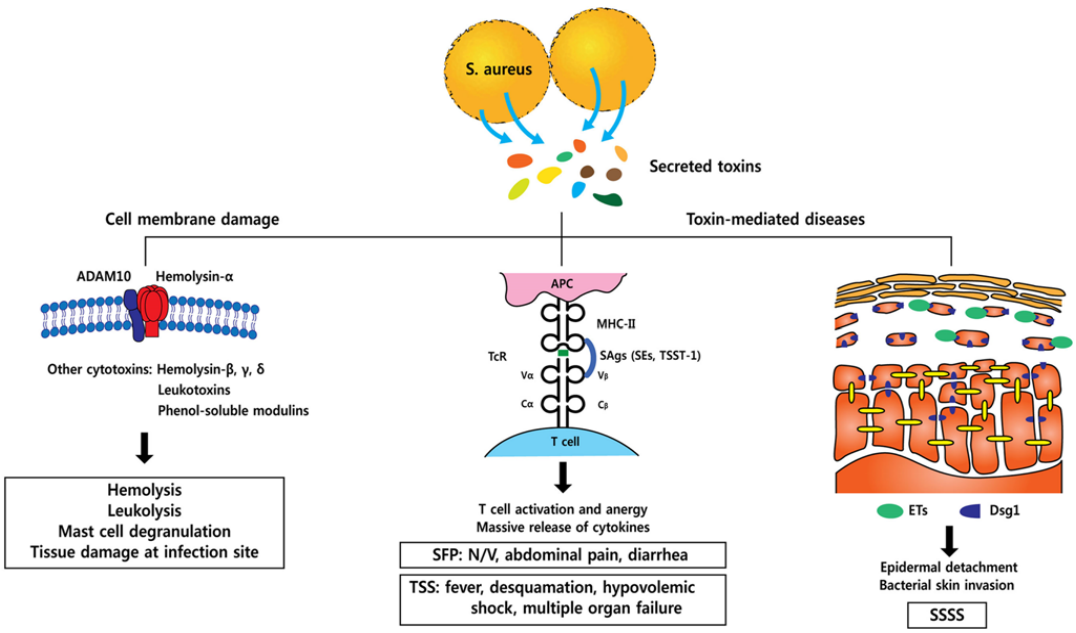


Figure 4: Schematic diagram of extracellular proteins produced by *S. aureus*. Their target and effect on cell structure: (on the left) *S. aureus* cytolytic toxins cause cell membrane damage and result in hemolysis. (in the middle) Staphylococcal super antigens eventually induce non-specific proliferation of T cells; intoxication by staphylococcal enterotoxins lead to staphylococcal food poisoning. (on the right) cell-cell adhesion disruption through desmoglein-1 hydrolysis by exfoliative toxins cause SSSS (Kim, 2019)

2.5. General aspects of virulence factors regulation

The large variety of virulence factors produced by *S. aureus* and their consistency in defining the course of staphylococcal infection with such fine orchestrated manner is mostly due to the complex regulatory system of the bacterium.

Several regulatory systems have been identified in the control of virulence factors production. The accessory gene regulator (*agr*) is a Two-Component System (TCS) of regulation which allows the pathogen to sense and respond to changes in environmental

conditions (Haag & Bagnoli, 2015). The *agr* system uses a quorum-sensing machinery to regulate exotoxin production in a cell density dependent pattern (Becker, 2018). Despite the somehow complex architecture of the *agr* quorum-sensing system, the functioning mechanism can be summarized as follows: (i) *S. aureus* synthetize a peptide called auto inducing peptide (AIP); (ii) as bacteria grow and reach a certain density in the environment, thus a certain concentration of AIP, the quorum-sensing system is triggered, activating the quorum signaling cascade; (iii) the resulting regulatory response can either promote or repress the expression of any of the virulence factor under the control of the regulatory system (Kong et al., 2016). The regulatory system controls several virulence factors, therefore plays an important role in pathogenesis. Studies in animal models have shown that *agr*-defective mutants show decreased severity of infection and lower levels of expression of toxins and factors in comparison to wildtype strains (Gong et al., 2014; Grundstad et al., 2019; Jenul & Horswill, 2019).

Additionally, there is the *SaeRS*, another TCS of *S. aureus*, which has been demonstrated to control the expression of virulence factors such as: *hla, Luk ED, coa, hlgABC in vivo*(Guo et al., 2017). Assisting biofilm formation and invasion of the host, interaction between *SaeRS* and *agr* has been a controversial point widely discussed in the literature and is also thoroughly described in a systematic review (Q. Liu et al., 2016).

3. Epidemiology

3.1. Carriage

Bacteria are not always pathogenic in the strictest sense. Understanding *S. aureus* pathogenesis requires insights into its mode of transmission and colonization. *S. aureus* is a commensal inhabitant of human epithelial surfaces, as well as a proven cause of wide range of infections (Tong et al., 2015). To successfully establish colonization, *S. aureus* needs to interact with human cell surface components through its expressed adhesives molecules (Krismer et al., 2017; Mulcahy & McLoughlin, 2016). *S. aureus* may inhabit the whole nasal area with anterior nares appearing to be the most prevalent site the organism can be isolated from (Kaspar et al., 2016). Variations of carriage rate in

individuals are observed according to the age, gender, geographic area, gender, part of the body colonized, and also host genetic determinants (Sollid et al., 2014). For example, higher carriage rates have been described in groups of HIV-infected individuals, in comparison to HIV-uninfected individuals (44% and 24% respectively) (Kotpal et al., 2016).

According to the 'culture rule' described by Nouwen et al in 2014, carriers may be classified into three groups: persistent carriers, intermittent carriers and non-carriers (Nouwen et al., 2004). Approximately 20% of the human individuals are permanent asymptomatic carriers, 20% are non-carriers and the remaining 60% are intermittent carriers of *S. aureus* (Laux et al., 2019). However, limitation of applied methodology encompasses previous research and current clinical practice screening which often typically investigate nasal colonization to rule out carriage. This may not reflect the real situation and further complicates our understanding of the significance of carriage state (Mehraj et al., 2016; van Belkum, 2016). The results presented by Senn et al. in 2016 describing a 'stealthy colonization of the gut' as an unrecognized niche, support that hypothesis (Senn et al., 2016).

Nevertheless, carriage of MRSA is an emerging and well acknowledged risk factor due to the antibiotic administration pressure that selects resistant strains (Lindsay, 2013).

3.2. Transmission

The high prevalence of carriage, either persistent or intermittent, combined with the asymptomatic nature render prevention and treatment of infections more challenging, particularly concerning MRSA. Person to person transmission constitutes the most important mode of transmission **(Figure 5)**. In healthcare facilities, healthcare workers, medical devices, patients and their relatives may be carriers of *S. aureus* including MRSA (Chang et al., 2017; Lamanna et al., 2017). Those carriers represent a source of transmission since they return back to community, where infection control measures are minimal, hence increasing community-based transmission (Barcudi et al., 2020; Lucet et al., 2009).

Similar work on transmission dynamics have indicated that worldwide dissemination on CA-MRSA carried by long distance traveling was strongly related to travel-associated MRSA skin and skin structure infections (Nurjadi et al., 2019). Moreover, isolation of *S. aureus* in neonates has been strongly correlated with whether the mother is a carrier or not (Lin et al., 2018). The 'cloud phenomenon' in *S. aureus* nasal carriers, is characterized by the air borne dispersal of the pathogen; although rare, this phenomenon has been described, and appear to be exacerbated in carriers suffering from viral upper respiratory infection (Bassetti et al., 2005; Bischoff et al., 2006).

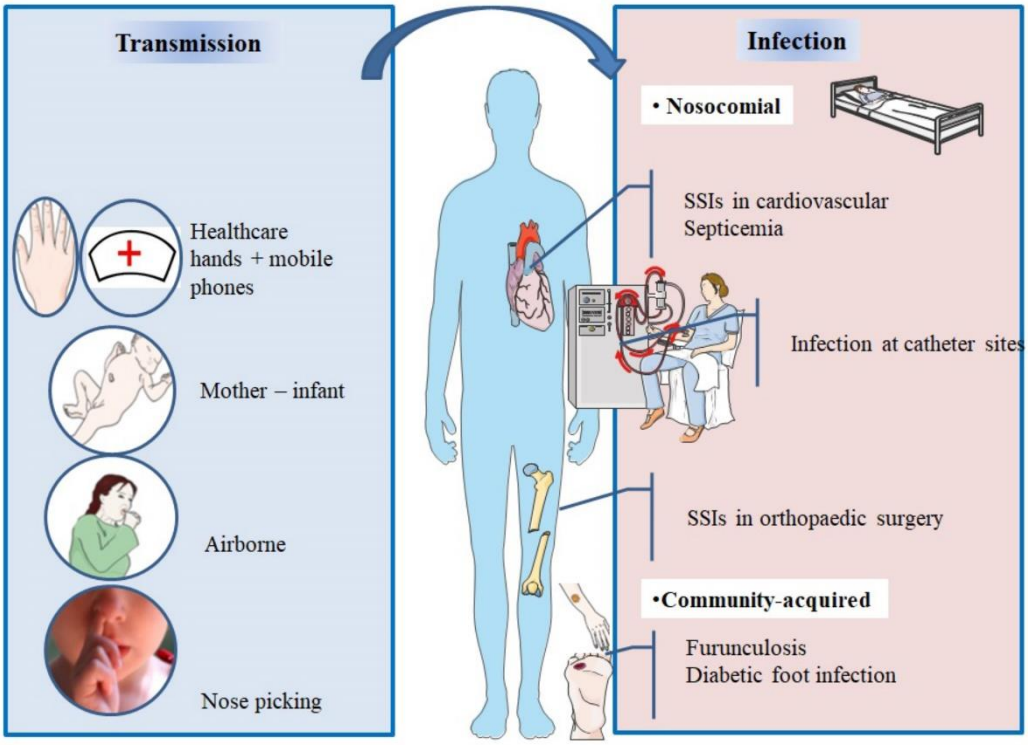


Figure 5: Schematic diagram of *S. aureus* transmission dynamic and possible related impact on healthcare and community (Sakr et al., 2018)

3.3. Risk factors

A quick review of the related literature allows to draw a conclusion: overall likelihood to develop opportunistic infection increases with tissue barrier disruption, combined to carriage and exposure to health care.

It has been reported that despite all measure taken prior operative surgery, *S. aureus*-induced surgical site infections is a recurrent cause of nosocomial infection (Pal et al., 2019). There is ample evidence indicating that preoperative topical decolonization as well as antibiotic prophylaxis decrease the risk of subsequent infection in patients (George et al., 2016; Lefebvre et al., 2017; Mallet et al., 2018; Septimus & Schweizer, 2016). With focus on intensive care unit (ICU) patients, Gagnaire et al. in 2019 pointed out the important role of *S. aureus* nasal carriage as a major risk of *S. aureus* ICU-acquired infection (Gagnaire et al., 2019). Moreover, infections, complication and mortality were significantly higher among individuals with decreased immune status who are prone to have higher carriage rate (Kotpal et al., 2016; Singla et al., 2019).

Most of the current evidence identifies intravascular and indwelling devices, medical biomaterials carriers as another groups subject to foreign-body related infections (Christensen et al., 2018; Matuszewska et al., 2020; Ricciardi et al., 2018; Sato et al., 2017).

3.4. Pathophysiology and clinical syndromes

Although staphylococcal infection may present diverse clinical foci, a consistent pattern can be derived in regard of the pathogenesis: (i) asymptomatic colonization of the skin and/or mucosa, as previously discussed, is the milestone for further infections; (ii) localized infections subsequent to trauma provide the pathogen with a route of entry to normally sterile body sites; (iii) given the extended armamentarium of *S. aureus*, hematogenous spread from primary foci is often seen and lead to multiorgan involvement and life threatening conditions (Tong et al., 2015). This section will briefly review some of the most important clinical manifestations.

3.4.1. Skin and skin structure infections

Previously known as skin and soft tissue infections, SSSIs are the most common manifestation of staphylococcal disease. Generally of variable presentation and severity, the etiology of SSSIs involves invasion of the skin and underlying tissues by the pathogen, following disruption of physical barriers; apart from trauma, special skin condition such as atopic dermatitis, increases the risk of developing secondary bacterial infection (Geoghegan et al., 2018; Hepburn et al., 2017).

SSSIs are most often associated with *S. aureus* as colonized patients are at higher risk for recurrent infection (Creech et al., 2015; Esposito et al., 2016). In 2018, while studying the effect of systemic antibiotics on *S. aureus* colonization and recurrent skin infection in children with SSSIs, Hogan et al demonstrated that 81% of *S. aureus*-SSSIs were caused by MRSA, with MRSA colonization in children as high as 63% (Hogan et al., 2018). Similar increase in the incidence of MRSA, particularly of CA-MRSA as a leading cause of SSSIs have been reported in many parts of the world (MacMorran et al., 2017). Importantly, PVL-positive strains were implicated, as the presence of PVL has clearly been associated with increased severity (Berla-Kerzhner et al., 2017; Klein et al., 2019).

SSSIs clinical presentation may vary from mild infections such as impetigo, ecthyma, to follicular infection such as folliculitis, furunculosis; and intradermal deep infections such as erysipelas, cellulitis, necrotizing fasciitis (Olaniyi et al., 2016).

3.4.2. Pleuropulmonary infection

Pneumonia is an inflammatory condition of the lung that occurs predominantly in children under the age of 5, and in individuals with underlying conditions (Masters et al., 2017). Two type of pneumonia can be presented: (i) community-acquired pneumonia that occurs without exposure to healthcare settings; (ii) healthcare acquired pneumonia, which comprises ventilator-associated pneumonia, and occurs 48 h after hospital admission or exposure to healthcare setting.

Necrotizing pneumonia is a severe invasive complication of pneumonia characterized by necrotic lung injury and abscess formation (Krutikov et al., 2019). The high incidence of this condition in children without any prior respiratory tract infection strongly support a consistent link between necrotizing pneumonia and community-acquired pneumonia, in particular involving CA-MRSA (Masters et al., 2017). CA-MRSA with necrotizing features have mostly been associated with PVL production and high mortality rate (Hayakawa et al., 2020; Takigawa et al., 2019). Moreover, superinfection with respiratory virus during a respiratory tract infection episode further complicates the clinical outcome. Bacterial proliferation is therefore, in this case, enhanced by virus induced immune modulation and cellular damage (Duployez et al., 2020).

In their study of hospital related pneumonia, Karatas et al. reported that *S. aureus* accounted for 15.1% of all ventilator-associated pneumonia cases (Karatas et al., 2016). Similar rate have been identified in Egypt (Galal et al., 2016) and in one Asian region meta-analysis (Bonell et al., 2019). Given the current SARS-CoV-2 pandemic, the MRSA challenge is to be considered to reduce the risk of superinfections (Lupia et al., 2020).

3.4.3. *S. aureus* bloodstream infections

Hematogenous spread of the bacteria may commonly originate from a primary clinical foci such as SSSIs, pneumonia, intravascular catheter (Sato et al., 2017); other risk determinants may be age, underlying condition or even ethnicity (Chaudry et al., 2019; McMullan et al., 2016). Bacteremia can remain uncomplicated, or evolve into complicated metastatic infections, such as infective endocarditis, osteomyelitis, septic arthritis (Horino & Hori, 2020).

3.4.4. *S. aureus* toxin mediated diseases

S. aureus toxin mediated diseases encompass: (i) *S. aureus* food-borne illnesses due to consumption of food contaminated with SAEs (Ondusko & Nolt, 2018); (ii) *S. aureus* toxic shock syndrome mediated by TSST and associated with either menstrual and

non-menstrual conditions (Tong et al., 2015); (iii) staphylococcal scalded skin syndrome (SSSS) is caused by ETs and mostly affects infants and small children's (Grama et al., 2016). Other clinical syndromes include meningitis, urinary tract infection and epidural abscesses. Although uncommonly caused by *S. aureus*, the implication of MRSA in these infections is increasingly reported and further complicates management of infections (Tong et al., 2015).

4. Antibiotic resistance in *S. aureus*

Antibiotic resistance in one of the biggest public health challenges of the era. From a genetic point of view, such resistance to antibiotics occurs upon changes in bacteria; considered as an adaptive response to the pressure of antibiotics. Basis of antibiotic resistance in bacteria have been extensively investigated for a considerable period, ranging from the early 20th century to current date. Regarding *S. aureus*, World Health Organization (WHO) Global Report on Surveillance of Antimicrobial Resistance 2014 clearly stated higher risk of death and higher additional cost (including antibacterial therapy and medical care cost) in patient suffering from an infection caused by a resistant strain.

Bacterial adaptation to environmental pressure of antimicrobial molecules, upon interaction with antimicrobial-producing microorganisms, is a widely observed phenomenon. Historically, two strategies have been used to tackle antimicrobial molecules effects; a) mutation, which unlike natural intrinsic resistance shared within a given species, arises spontaneously upon exposition to antimicrobial molecule (Munita et al., 2016); b) horizontal gene transfer, the second strategy, encompasses all the process leading to acquisition of foreign genomic material either by: (i) transformation by DNA uptake from the surrounding environment, (ii) transduction or acquisition of genomic material via a viral vector, (iii) conjugation, the bacterial sex, involving the exchange of genomic material between two bacteria (Lerminiaux & Cameron, 2019).

4.1. Mechanism of antibiotic resistance

The above-mentioned strategies generally affect antimicrobial molecule action either by: (i) decreasing affinity of the drug toward its target through target modification or target bypass; (ii) activation of drug efflux pump; (iii) modification of regulatory systems controlling genes encoding for drug transporters; (iv) degradation or modification of the antimicrobial compound (C Reygaert, 2018; Peterson & Kaur, 2018).

4.2. Antibiotics resistance in clinical environment

Although a major problem in clinical settings and associated with increased additional cost and mortality, antibiotic resistance, first to penicillin, then to methicillin and more recently to vancomycin, have been evidenced to be present in environmental microorganisms prior the introduction of the drugs (Harkins et al., 2017; Ogawara, 2016). The wave of resistance to methicillin that arose after its introduction to clinical use, started in 1961, with the first report of a methicillin resistant variant in *S. aureus* (BARBER, 1961).

This section reviews the successive wave of resistance in *S. aureus*, with particular emphasis on resistance to methicillin clonal diversification and current status of the challenge.

4.2.1. The wave of penicillin resistance

History credits Alexander Fleming with discovering penicillin in 1928 (Gaynes, 2017). Its introduction to clinical use later 1941 has led unfortunately to the emergence of penicillin resistant strains as early as 1942. First restricted to hospital settings, these strains became pandemic by spreading to community by late 1960s (Lowy, 2003).

S. aureus resistance to penicillin in mediated by the *blaZ* gene, encoding for a β-lactamase enzyme. Briefly, bacterial exposition to penicillin, and more generally β-lactam

antibiotics, activate the *blaZ* signal transducer results the transcription and the production of the β-lactamase enzyme which hydrolyses the β-lactam ring and renders the antibiotic inactive (Shalaby et al., 2020). As high as 99% of circulating *S. aureus* strains carry the *blaZ* gene and are resistant to penicillin (Vestergaard et al., 2019).

4.2.2. Penicillin binding proteins and PBP2a resistance

4.2.2.1 Penicillin binding proteins

The synthesis of bacterial cell wall is a complex process involving several steps and components. Penicillin-binding-proteins (PBPs) are membrane bound proteins that catalyze reaction of bacterial cell wall synthesis (carboxypeptidation and transglycosylation) during the extracellular chain cross-linking (Shalaby et al., 2020). There are four types of PBPs in *S. aureus*: PBP1, PBP2, PBP3 and PBP4 (Kirmusaolu, 2017).

4.2.2.2 PBP2a

Originally, PBP2 is a dual function enzyme that catalyze both transpeptidation and transglycosylation. Its inhibition leads to impaired peptidoglycan cross-linking and subsequent bacterial lysis through leakage of the cytoplasmic content. In the presence of β-lactam antibiotics, *S. aureus* strains resistant to methicillin produce a unique PBP in large amount and bacteria can still proliferate. The resistance basis is related to the acquisition of *mecA* gene, a resistance determinant carried by the *SCCmec*, which encodes for a novel PBP: PBP2a or PBP2'. PBP2a, co-functioning with other PBPs, is able to continue the peptidoglycan elongation as well as cross-linking, thus enables the bacteria to exhibit resistance to methicillin. The mechanistic of resistance is achieved by change in target; PBP2a exhibit a distortion of its active site and prevents β-lactams binding, while allowing peptidoglycan substrate to continue elongation (Shalaby et al., 2020).

4.2.2.3 Clinical impact of β-lactam resistance: MRSA

S. aureus is a worldwide cause of both community and hospital acquired infection as well as multidrug resistant infections (Henderson & Nimmo, 2018; Thai et al., 2019). Methicillin is a semi-synthetic β-lactamase-insensitive β-lactam. MRSA variant appeared in 1961, and rapidly spread worldwide (Lee et al., 2018). Since first identified among clinical isolates, a contemporary definition of hospital associated-MRSA emerged and define all *S. aureus* isolates recovered from patients 2 or more days after hospital admission or with MRSA risk factors such as antecedent of recent hospitalization, dialysis, surgery, indwelling medical device. Its counterpart, community associated-MRSA, define all *S. aureus* isolates recovered from patients within 2 days of hospital admission and without previously-mentioned MRSA risk factors (Gnanamani et al., 2017).

4.2.2.4 HA-MRSA, CA-MRSA and LA-MRSA

Since its emergence, MRSA has been a leading cause of hospital-associated nosocomial infections; those infections are increasingly difficult to treat due to their association with other antibiotics resistance traits phenotypically manifesting as multidrug resistant-MRSA (Parhizgari et al., 2016; Thai et al., 2019). From such challenge emerges new concerns, as those infections are related to longer hospital stay and increased economic burden (Thampi et al., 2015).

Given its remarkable fitness, the multitude of virulence factors secreted, its ability to reside as a human commensal and overcome antibiotic pressure, it is important to understand the interplay between risk factors. *S. aureus* associated infections represent a high proportion of infections in individuals with health-care associated risk factors (Mao et al., 2019). With nasal and skin carriage as first determinant risk factor for subsequent infection, it has interestingly been reported that bloodstream infection causative strain and nasal strain share the same genotype in most of the cases (Lakhundi & Zhang, 2018). A systematic review, reported that MRSA isolation was 55% higher in burn ICU patients (Khan et al., 2018). Although MRSA has diversified into a large variety of clones and

disseminated worldwide (Andrade-Figueiredo & Leal-Balbino, 2016; Aung et al., 2019; Zarfel et al., 2016), their distribution and their relative fitness within health care settings vary with geography (Lakhundi & Zhang, 2018).

Considered until 1990s as endemic to hospital settings, the epidemiology of MRSA has drastically changed with the emergence of CA-MRSA. CA-MRSA tends, conversely to its counterpart, to occur in healthy individuals, displaying SSSIs as the predominant clinical manifestation. Epidemiologically, CA-MRSA is most often associated with *SCCmec* type IV (Rebić et al., 2019; X. Wang et al., 2019). PVL has been closely regarded as a hallmark of CA-MRSA, and has been described as a promoter of condition severity (Berla-Kerzhner et al., 2017; Nurjadi et al., 2019; Olaniyi et al., 2016), although the role of the latter in pathogenesis has been subject to many controversies; some studies describe it as of high relevance (Berla-Kerzhner et al., 2017; Klein et al., 2019), yet its direct role in pathogenesis is still not clearly understood (Nawrotek et al., 2018).

The frontiers between CA- and HA-MRSA became blurry with the increasing observed occurrence of CA-MRSA causing invasive, nosocomial infections; following the current trend, CA-MRSA tend to invade healthcare settings and in the future replace HA-MRSA as the dominant variant (Choo, 2017; Kateete et al., 2019; Klein et al., 2019; Lamanna et al., 2017). Moreover, although it appears to be limited to β-lactam resistance (Lakhundi & Zhang, 2018), genetic diversification may lead to the acquisition of additional antimicrobial resistance traits in CA-MRSA.

In addition to what have been discussed in this section, it is noteworthy to mention a variant of MRSA related to livestock's that have transferred to humans. Livestock's have been recognized as a reservoir for MRSA colonization in humans. Reported cases of human colonization by MRSA originated from animals have been described. In Germany, a study reported that 10%, 15% and 3% of MRSA isolated respectively from septicemia, wound infection and general nosocomial infection were found to be livestock-associated MRSA (LA-MRSA) (Cuny et al., 2015). Individuals with animal occupational exposure also appeared to have higher carriage rate (77% to 86%) in comparison to normal population, and interestingly, LA-MRSA associated infections reported were likely to occur in areas of farm settings. Consistently supporting previous findings, a comparative

study of both HA-MRSA and LA-MRSA described less virulence in the latter; antibiotic susceptibility pattern were also different and dependent on whether a given antibiotic was extensively used in either human or animal health management (Mutters et al., 2016). It appears that LA-MRSA asymptomatic carriers could be involved into the spread of LA-MRSA among farm settings and urban areas (Larsen et al., 2016) leading to an increased incidence of LA-MRSA in hospital settings (Kevorkijan et al., 2018). This epidemiological shift complicates the management policies designed against hospital and community acquired MRSA spread.

5. Staphylococcal α-toxin

5.1. General overview

α-toxin is the most studied member of staphylococcal hemolysins. Its role in pathogenesis of staphylococcal infections is critical and most often associated to hemolytic, dermonecrotic and lethal activities (Berube & Wardenburg, 2013). Also known as *hla* or α-hemolysin, α-toxin is the prototypic β-barrel pore forming toxin. The toxin is encoded by the *hla* gene, part of the bacterial genome, and is secreted as a 33 kDa water soluble monomer (Oliveira et al., 2018). Upon binding on host cell membrane, α-toxin monomers oligomerize into heptameric β-barrel pore across the membrane of the target cell, thus form a transmembrane channel (Tam & Torres, 2019). The toxin, found to act upon a large variety of cell and species is of increasing relevance since *hla* gene and toxin expression is highly conserved in *S. aureus* strains; 99.6% and 97.6% for MSSA and MRSA respectively, according to the findings of Tabor and colleagues (Tabor et al., 2016). α-toxin expression in tightly controlled by several regulatory systems; the accessory gene regulator (*agr)*, the staphylococcal accessory gene regulator (*sarA)*, the staphylococcal accessory protein effector (*sae*), the repressor of proteins regulatory protein (*rot)*, among others have been described (Jenul & Horswill, 2019).

5.2. Toxin structure

As previously mentioned, α-toxin is secreted as a water-soluble monomer and oligomerizes on the host cell membrane. Early studies of the toxin structure have used electron micrograph as well as purified membrane bound α-toxin pore and described the stand-alone as a circular ring-like structure (von Hoven et al., 2019). High resolution crystallography characterized the mushroom-like structure of α-toxin describing three different domains: (i) cap domain, the entry on the pore, on the extracellular face; (ii) rim domain, the pore interface embedded in the target cell membrane and (iii) stem domain, the membrane perforating channel that runs across the host cell membrane **(Figure 6)**.

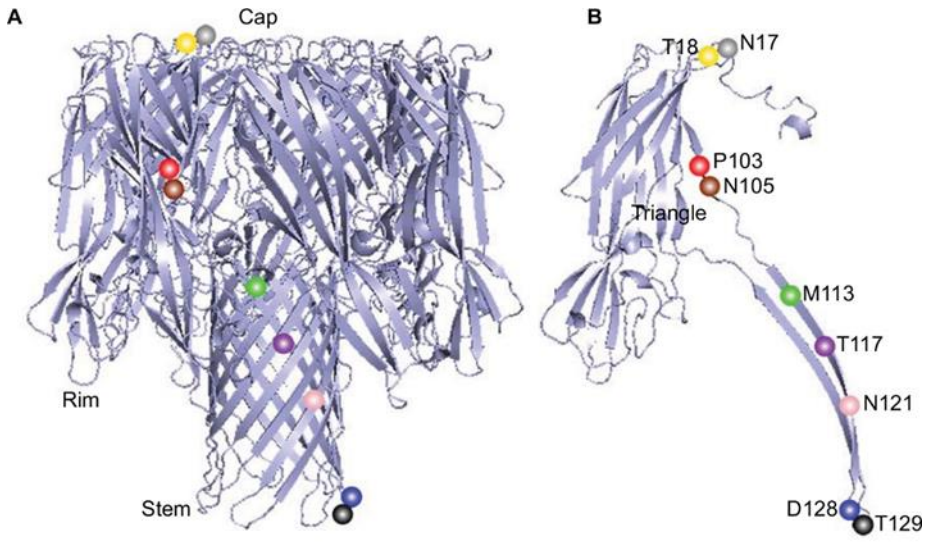


Figure 6: α-hemolysin molecular structure. (A) The heptameric mushroom-like structure showing three domains; the cap, extracellular domain; the rim, interfacing domain and the stem, transmembrane channeling domain. (B) The monomeric form of α-hemolysin. (Du et al., 2018)

5.3. α-toxin binding to target cells

A disintegrin and metalloprotease 10 (ADAM10) has been identified as the receptor of α-toxin on host cell membrane (Wilke & Wardenburg, 2010). Although the heptameric oligomer formation mechanism is yet to be resolved, the role of ADAM10 in α-toxin-mediated toxicity have been extensively studied (**Figure 7**) (von Hoven et al., 2019).

Using human keratinocytes cell line, Von Hoven and colleagues confirmed ADAM10 as a promoter of α-toxin binding, and related ADAM10 enzymatic activity toward E-cadherin as an α-toxin dose-dependent activity (Von Hoven et al., 2016). Additionally, the authors have (as well as Power and colleagues in 2015 that have used a knockout approach of ADAM10 in platelets) showed that specific knockout of ADAM10 renders HAP1 cells α-toxin-insensitive, in comparison to parental HAP1 cells (Powers et al., 2015; Von Hoven et al., 2016). There is therefore ample evidence supporting ADAM10 as the high affinity receptor for α-toxin. Moreover, several studies have evidenced a complementary co-functioning between ADAM10 and other membrane proteins as directing sensitivity to α-toxin and cellular observed effect of ADAM10-α-toxin interaction (Popov et al., 2015; Shah et al., 2018; Winter et al., 2016).

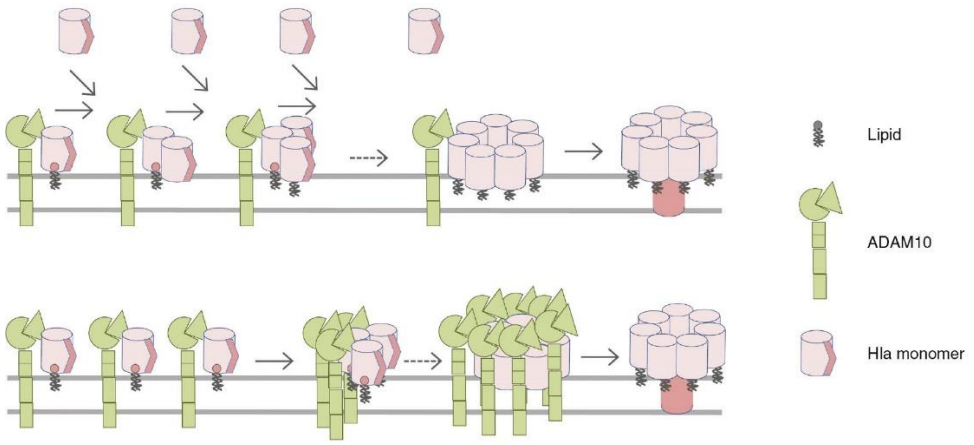


Figure 7: Model illustrating key functions of the α-toxin (monomer)-ADAM10 (green) complex, facilitating membrane binding of the toxin with subsequent oligomerization and pore formation (von Hoven et al., 2019)

5.4. Post binding signaling

Tissue barrier disruption is a hallmark of staphylococcal infections. This mechanism can be clearly understood when the sheddase activity of ADAM10 is taken in account. Indeed, ADAM10-induced cleavage of E-cadherin and/or its clustering with PLEKHA7 or other membrane proteins results in focal adhesion disruption and characteristically manifest as epithelial damage in skin, lung or vascular endothelium lesions.

Multiple studies have described the important role of α-toxin invasive infections. Jenkins and colleagues reported an upregulation of *hla* gene in pathogenic invasion process in bacteremia and sepsis model, compared to the nasal colonization model in mice (Jenkins et al., 2015). Using murine skin infection model, it has been evidenced that α-toxin contributes to delayed wound healing by impairing *S. aureus* clearance and promoting bacterial evasion (Falahee et al., 2017; Goldmann et al., 2016; Putra et al., 2019) and α-toxin hyper expression has been associated to increased virulence (Chua et al., 2014).

Of note, α-toxin-induced inflammation implies a modulation of host immune system; one of the immediate consequences of the pore formation across the membrane is ion exchange. On purpose: (i) K^+ ions efflux through α-toxin pore, and production of IL-1β by macrophages in the local environment, both sensed by node-like receptor (NLRP3), result in the activation of the inflammasome (Gaidt & Hornung, 2018; von Hoven et al., 2019); (ii) rapid influx of Ca^{2+}, an important intracellular signal mediator, triggers several cell-death pathways (Bouillot et al., 2018). Intravascular endothelium impairment through ADAM10- α-toxin interaction, as well as impeded endothelial repair via platelet aggregation and exacerbated host inflammatory response causing liver injury during sepsis have been also reported as α-toxin-induced consequences (Powers et al., 2015; Surewaard et al., 2018).

6. Staphylococcal exfoliative toxins

Exfoliative toxins are potent serine proteases which possess protease activity (hydrolysis of desmoglein-1, a cell adhesion molecule), cleave desmosomal cell attachment, and are related to cell-cell adhesion disruption in keratinocytes, resulting in exfoliation and blistering (**Figure 8**) (Kim, 2019). In *S. aureus*, there are four antigenically distinct exfoliative toxins in *S. aureus*: ETA, ETB, ETC, ETD (Tam & Torres, 2019); however, only exfoliative toxin A and B (ETA and ETB), two related, yet immunologically distinct serotypes, have particularly been strongly associated with human Staphylococcal Scalded Skin Syndrome (SSSS) (Grama et al., 2016; Staiman et al., 2018) as they present identical dermatologic symptoms (Mariutti et al., 2017). The associated etiology, SSSS, a blistering affection of the epidermis, may take have two different presentations: (i) the bullous impetigo, a localized form, may occur at any age; (ii) conversely, the generalized form which predominantly occurs in neonates and is thought to be due to the underdeveloped immune system and ETs absorption from a primary localized foci and diffusion through bloodstream (Bukowski et al., 2018; Staiman et al., 2018).

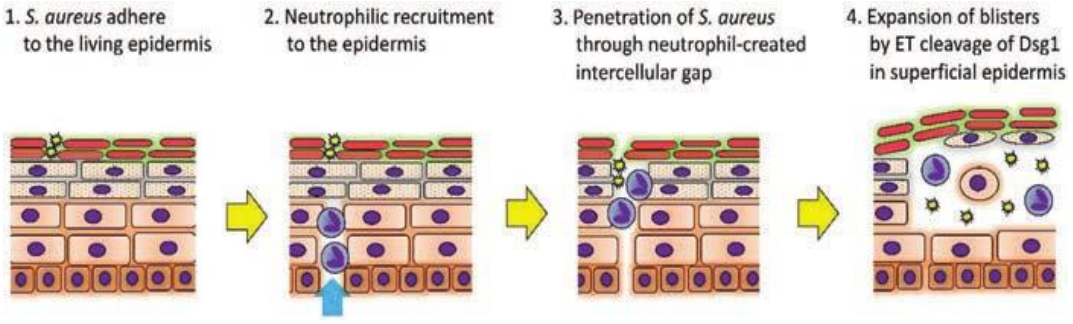


Figure 8: Pathogenesis of cutaneous invasion and blistering (Mariutti et al., 2017)

Epidemiological data on ET-producing *S. aureus* are very variable. ETA (1.5%) and ETB (0.5%) appear to be the most predominant serotypes (Bukowski et al., 2018). However, geographic differences have been shown regarding the incidence of strains producing either ETA alone, ETB alone or both ETA and ETB. ETA appears to be the

dominant serotype in Europe, USA, and Africa whereas ETB is mostly characterized in Japan. Recently, epidemic strain of ETA-producing *S. aureus* has been identified as the cause of skin and soft tissue infection outbreak in neonates (Pimentel de Araujo et al., 2018). A systematic review and meta-analysis in Iran revealed that ETA and ETB were present in respectively 13.05% and 3.6% of *S. aureus* isolates (Memariani et al., 2020). Amirmozafari and colleagues have found a high prevalence of ETA in particular, in both MRSA and MSSA (Amirmozafari et al., 2019).

SECTION TWO: MATERIALS AND METHODS

2.1. Bacterial isolates

In this study, 94 *S. aureus* isolates obtained from patients admitted to Near East University Hospital between January 2012 to November 2020 were randomly selected and included in this study. Isolates were processed by subculturing onto blood agar plates to obtain pure cultures at Near East University Hospital Microbiology Laboratory. Bacterial identification as *S. aureus* and resistance to methicillin was initially determined using coagulase tube test and BD Phoenix 100 automated identification and antibiotic susceptibility system (Becton-Dickinson Diagnostic).

Isolates were collected from both inpatient and outpatient group and were recovered from various clinical foci including wound/abscess, blood, broncho-alveolar lavage, nasal swab, tracheal aspiration and sputum.

2.2 Coagulase tube test

Individual bacterial colonies were mixed into 1 ml of human plasma. The samples were then incubated at 37°C for 4 h. The samples were reported as positive to coagulase if there were clot formation after the incubation period, thus indicating the presence of *S. aureus.* In the absence of clots after the incubation period, the samples were reported coagulase negative staphylococci **(Figure 9)**.

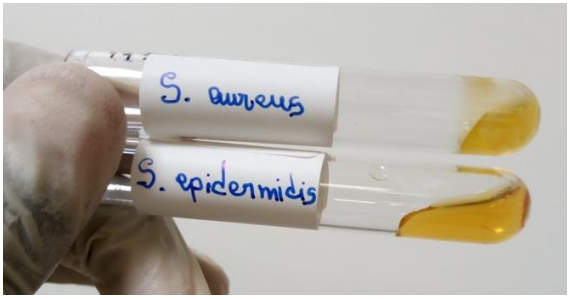


Figure 9: Coagulase tube test. (top) *S. aureus* showing coagulation after 4h of incubation at 35°C; (bottom) *S. epidermidis*, coagulase negative staphylococcus

2.3 Antibiotic susceptibility testing

Resistance to cefoxitin has been confirmed as a surrogate marker for screening of resistance to methicillin in *S. aureus* (Kriegeskorte et al., 2017). In addition to the previous antibiotic susceptibility results from the BD Phoenix 100, all isolates were subjected to cefoxitin disc diffusion test as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committe on Antimicrobial Susceptibility Testing, 2020). Testing was carried out on Mueller-Hinton agar plates which were prepared according to the manufacturer instructions. Plates were then inoculated with each isolate using direct colony suspension method by preparing suspension of the micro-organism in saline to the density of 0.5 McFarland turbidity standard.

Antimicrobial disc containing cefoxitin (30µg) were then applied and plates were incubated aerobically at 35°C for 24h. After incubation, interpretation of susceptibility was made as per EUCAST guidelines (EUCAST, 2020). Measurement of the inhibition zone edge made with transmitted light, *S. aureus* isolates were reported respectively as methicillin susceptible if zone diameter >22mm; and as methicillin resistant if zone diameter <22mm **(Figure 10)**.

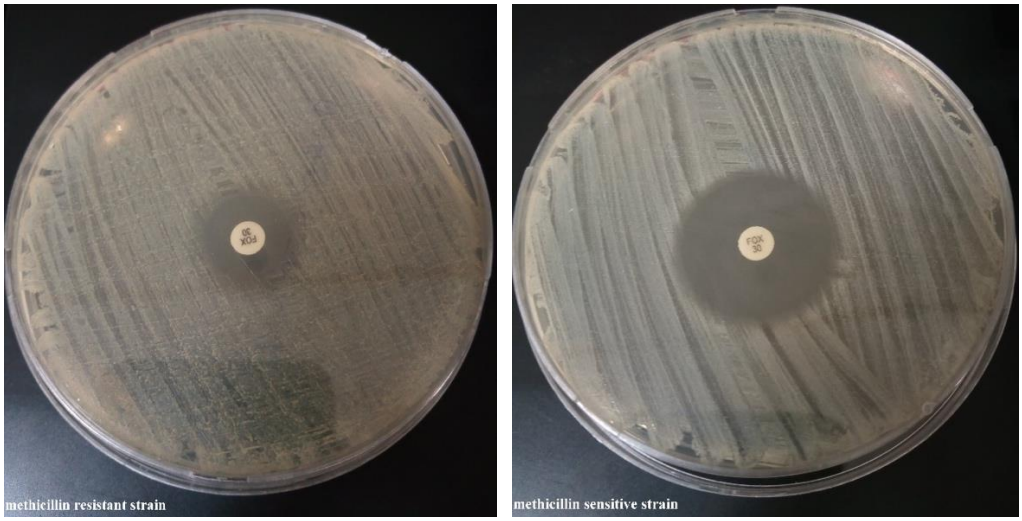


Figure 10: Antibiotic susceptibility testing on Mueller-Hinton agar

2.5 DNA extraction for molecular tests

All isolates were cultures on blood agar and incubated overnight at 37°c. The DNA isolation was carried out by heat extraction (boiling method) as described by Barbosa et al in 2016 (Barbosa et al., 2016) with modifications. Briefly, portions of few colonies were suspended into eppendorf tubes containing 500 µl of sterile water. The cell suspensions were incubated at 100°C for 15 min in a heat block. The tubes were centrifuged at 13000 rpm for 10 minutes to sediment the debris and the supernatant. The supernatant containing bacterial DNA was transferred to sterile tubes and were stored at -20°C until needed for PCR amplification.

2.6. PCR amplification of *nuc, hla* and *exfa* genes

All isolates identified as MRSA were subsequently screened for the presence of the staphylococcal thermonuclease gene (*nuc*) specific for *S. aureus* and the following virulence factor genes: staphylococcal α-toxin gene *(hla)* and exfoliative toxin A gene *(exfA).* The sequence of primers used in this study and the size of the amplicon are given in Table 1

Each 25 µl PCR reaction contained: 12.5µl of 2x PCR master mix (Thermo Scientific); 1 µl of both forward and reverse primers (at a final concentration of 10pmol/µl) for the target genes (respectively *nuc*, *hla* and *exfa*); 6.5 µl of nuclease free PCR grade water (Thermo Scientific) and 4 µl of DNA template. Table 2 reports the cycling conditions used for each single PCR amplification.

S.aureus SCC mec type IV was used as positive control for both *nuc* and *hla* gene amplification. Nuclease free PCR grade water served as negative control for all the amplification reactions. All primers sequences used in this study have been obtained from previously published studies; *nuc, hla* and *exfa* primer sequence were respectively used by Hanssen et al in 2004, Shekarabi et al in 2017 and Suryadevara et al in 2013 (Hanssen et al., 2004; Shekarabi et al., 2017; Suryadevara et al., 2013)

Table 1: Sequence of primers used for PCR amplification

Gene	Primer sequence (5′ to 3′)	Product size (bp)
nuc-F	GCG ATT GAT GGT GAT ACG GTT	279 bp
nuc-R	AGC CAA GCC TTG ACG AAC TAA AGC	
hla-F	CTG ATT ACT ATC CAA GAA ATT CGA TTG	209 bp
hla-R	CTTT CCA GCC TAC TTT TTT ATC AGT	
exfa-F	GCA GGT GTT GAT TTA GCA TT	93 bp
exfa-R	AG ATG TCC CTA TTT TTG CTG	

Table 2: PCR cycling conditions used for each amplification

Genes	Steps	Temperature	Time	Cycles
nuc	Initial denaturation	94°c	10 min	1
	Denaturation	94°c	30 secs	35
	Annealing	57°c	30 secs	
	Extension	72°c	90 secs	
	Final extension	72°c	10 min	
	Hold	4°c	-	1
hla	Initial denaturation	95°c	10 min	1
	Denaturation	94°c	1 min	35
	Annealing	57°c	1 min	
	Extension	72°c	1 min	
	Final extension	72°c	7 min	
	Hold	4°c	-	1
exfA	Initial denaturation	95°c	10 min	1
	Denaturation	95°c	1 min	35
	Annealing	58°c	1 min	
	Extension	72°c	1 min	
	Final extension	72°c	10 min	
	Hold	4°c	-	1

2.7 Agarose gel electrophoresis

The amplified products (11µl) were mixed with 3.5 µl of loading dye and were electrophoresed in 2% agarose gel (W/V) prepared with 1x Tris Borate EDTA (TBE) buffer; ethidium bromide was used as a fluorescent tag at a concentration of 0.5µg/ml. A 50 bp DNA ladder was used as DNA molecular weight marker. The separated DNA fragments were visualized and imaged under UV light on a transilluminator (DNA MiniBIS Pro Gel Imaging System.).

SECTION THREE: RESULTS

3.1 Patient and sample characteristics

In this study, a total of 76 confirmed MRSA samples collected from patients admitted to Near East University Hospital were investigated. Among the patient groups, forty-four (57.89%) patients were male and thirty-two (42.1%) were female. The patient's age at admission varied from 1 to 99 years with a median at 60 years; details of patient's distribution by gender and age group are shown in Figure 11.

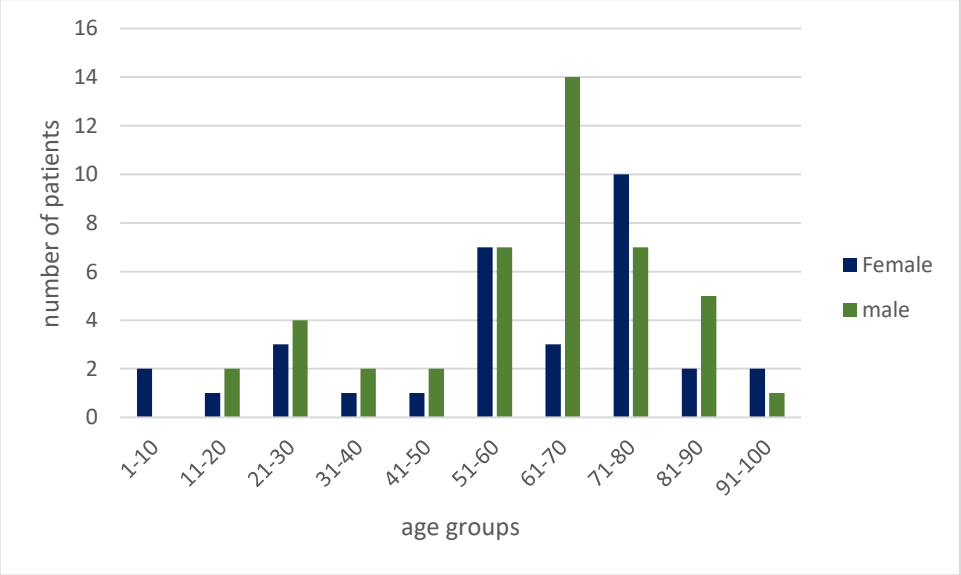


Figure 11: Distribution of patients by gender and age group

Overall MRSA cases occurred in 75% (n=57) of the cases among inpatients, while outpatient MRSA infections accounted for the remaining 25% (n=19). Distribution of MRSA cases by admission type is presented in Figure 12.

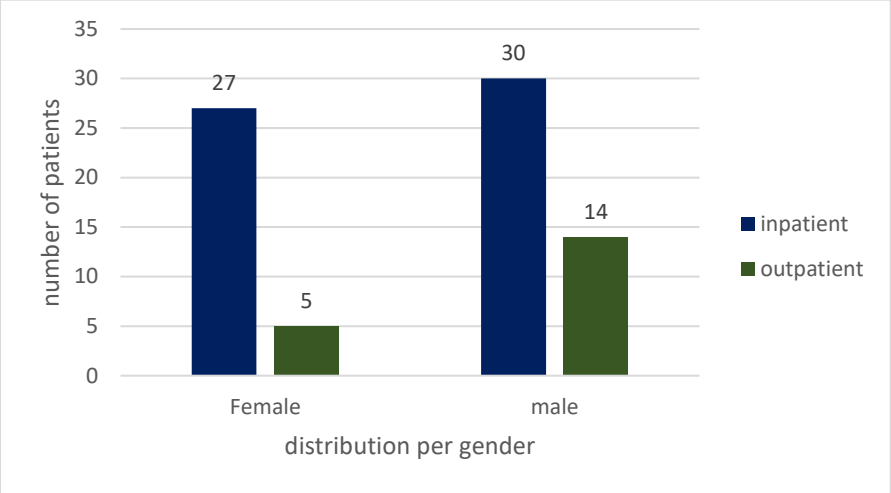


Figure 12: Distribution of cases in inpatient and outpatient groups by gender

Clinical samples were obtained from patients admitted to various departments including: Cardiology (n=14; 18.4%), Pulmonary Infections (n=10; 13.2%), Laboratory (n=8; 10.5%), Anesthesia (n=7; 9.2%), Orthopedics (n=6; 7.9%), both Cardiovascular Surgery, General Surgery and Neurosurgery (n=5; 6.6%), Dermatology (n=4; 5.3%), both Brain Surgery, Gastroenterology and Intensive Care Unit (n=2; 2.6%), and the remaining departments: Geriatrics, Dialysis, Plastic Surgery, Urology, Infectious Disease (n=1; 1.3%) **(Figure 13).**

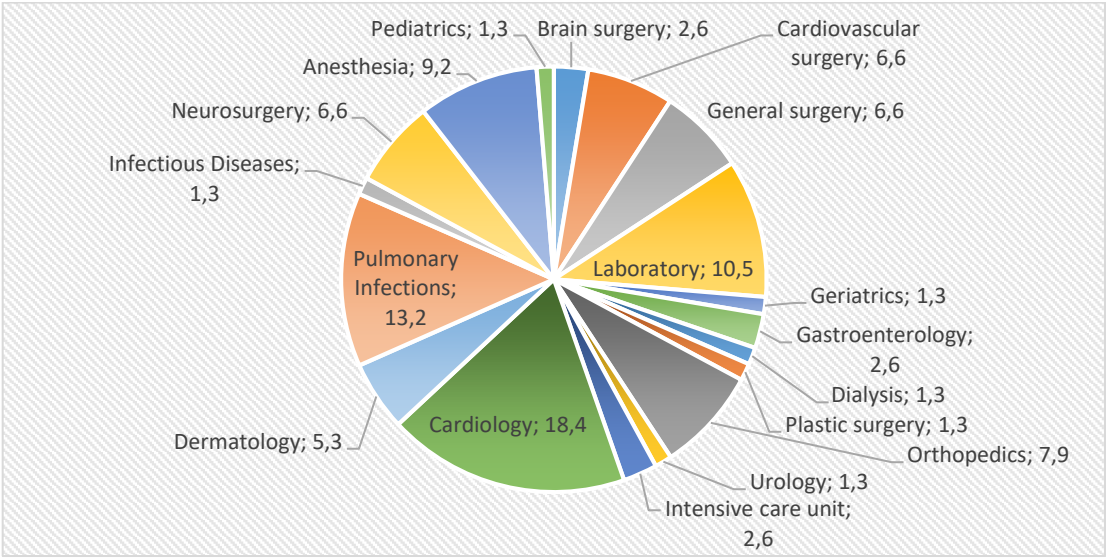


Figure 13: Distribution of samples by hospital departments

When the distribution of the isolates according to the clinical samples was considered, the isolates were found to be isolated from blood (n=17; 22.4%), abscess/wound (n=19; 25%), tracheal aspirate (n=13; 17.1%), urine (n=4; 5.3%), nasal swab (n=13; 17.1%), catheter tip (n=3; 3.9%), sputum (n=5; 6.6%), bronchoalveolar lavage and urethral sample (n=1; 1.3%) **(Figure 14)**.

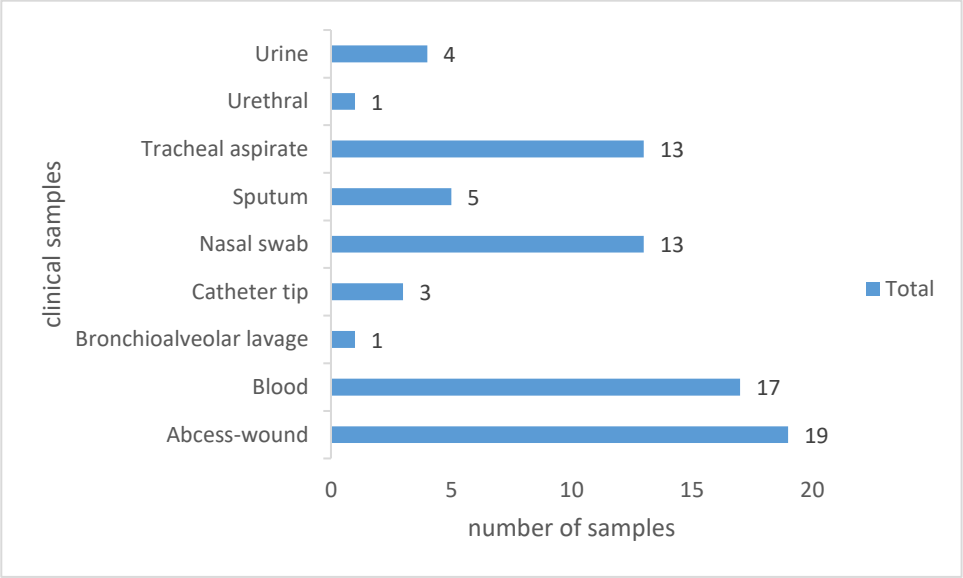


Figure 14: Distribution of isolates according to the clinical samples

3.2 Phenotypic characterization of MRSA

Among the total of 94 *S. aureus* isolates, 80.85% (n=76) were confirmed as MRSA using coagulase tube test and cefoxitin disc diffusion assay.

3.3 Amplification of *nuc, hla* and *exfA* genes

S. aureus specific *nuc* gene encode for the thermostable nuclease of *S. aureus*. Species identification of all isolates were confirmed using PCR amplification of the *nuc* gene. 100% (n=76) of the *S. aureus* isolates presented with the expected amplicon size of 270 bp shown in Figure 15. The presence of *hla* and *exfA* virulence genes was detected respectively in 97.36% (n=76) and 2.63% (n=2) of the samples.

For all PCR amplifications performed, there were no other non-specific band, and the amplicon size for *hla* and *exfA* genes were respectively 209 bp and 93 bp, as shown in Figure 15, Figure 16 and Figure 17 respectively.

nuc gene amplification images

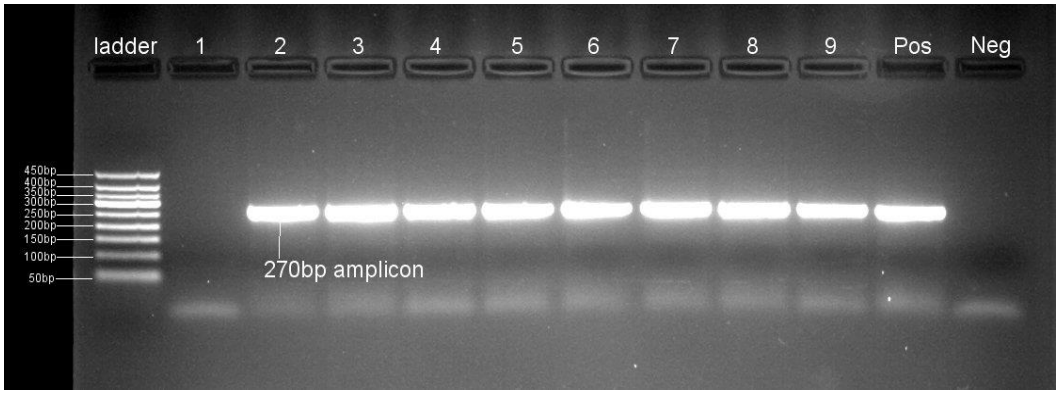
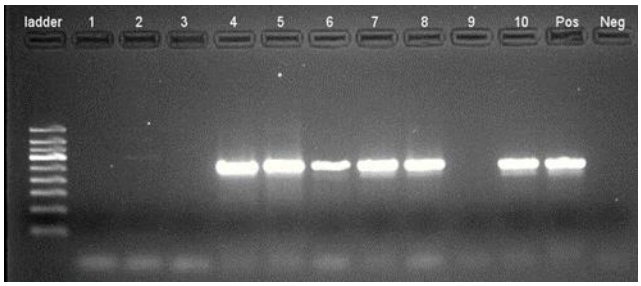
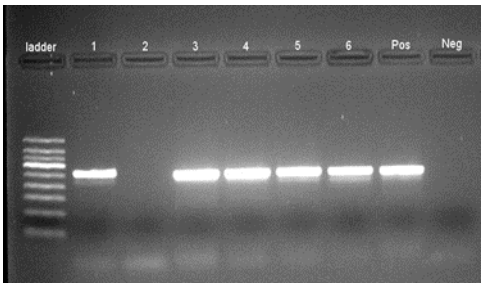
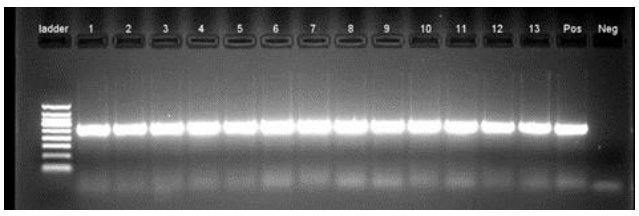


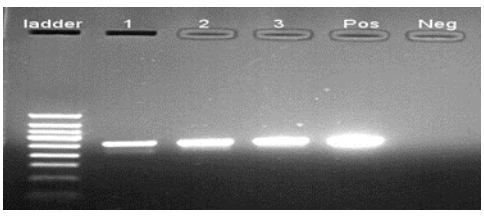
Figure 15: PCR showing positive amplification of 270 bp fragments specific for *nuc* gene of *S. aureus.* Lines: 'ladder', DNA molecular weight marker 50bp; 1, negative isolate; 2 to 9, positive isolates; 'pos', positive control; 'neg', negative control.

Following images show amplification of *nuc* gene (270 bp amplicon) in remaining clinical samples with a 50 bp DNA molecular weight marker.









***hla* gene amplification images**

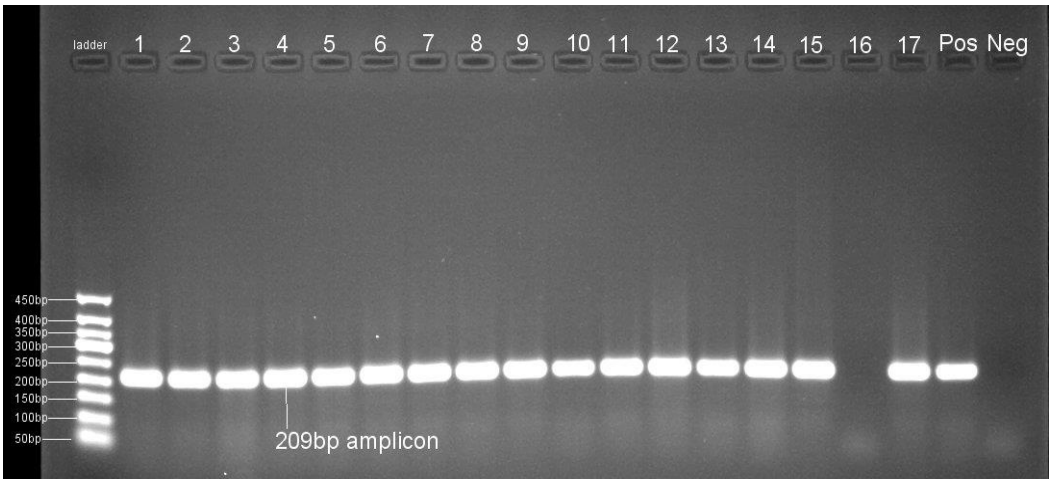
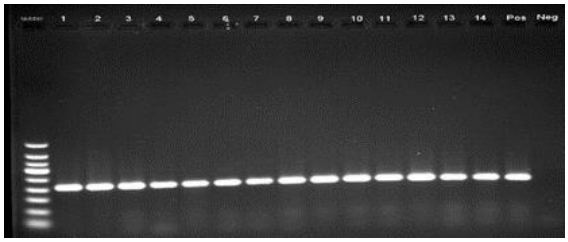
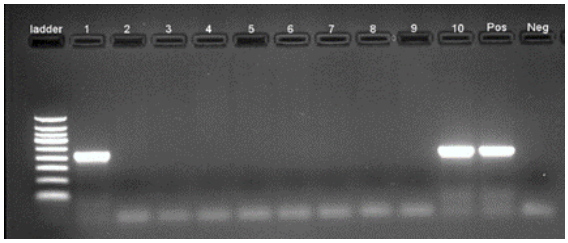
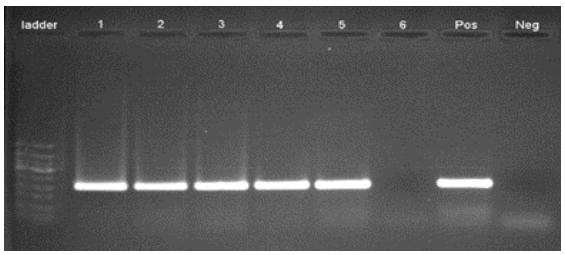


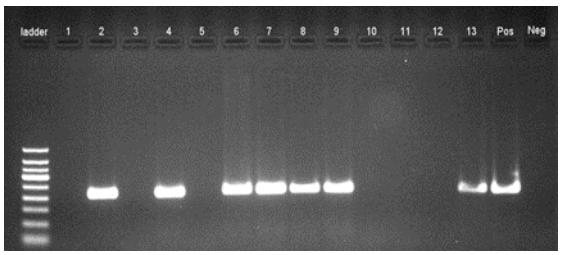
Figure 16: PCR showing positive amplification of 209 bp fragments specific for *hla* gene of *S. aureus.* Line: 'ladder', DNA molecular weight marker 50bp; 1 to 15 and 17, positive isolates; 16, negative isolate; 'pos': positive control; 'neg': negative control.

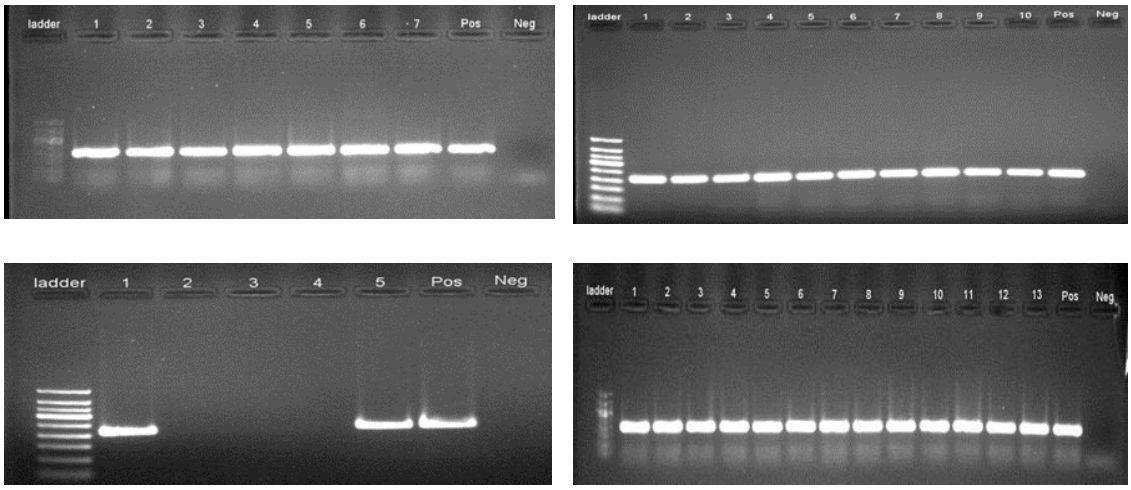
Following images show amplification of *hla* gene (209 bp amplicon) in the remaining clinical samples with a 50 bp DNA molecular weight marker.











***exfa* gene amplification images**

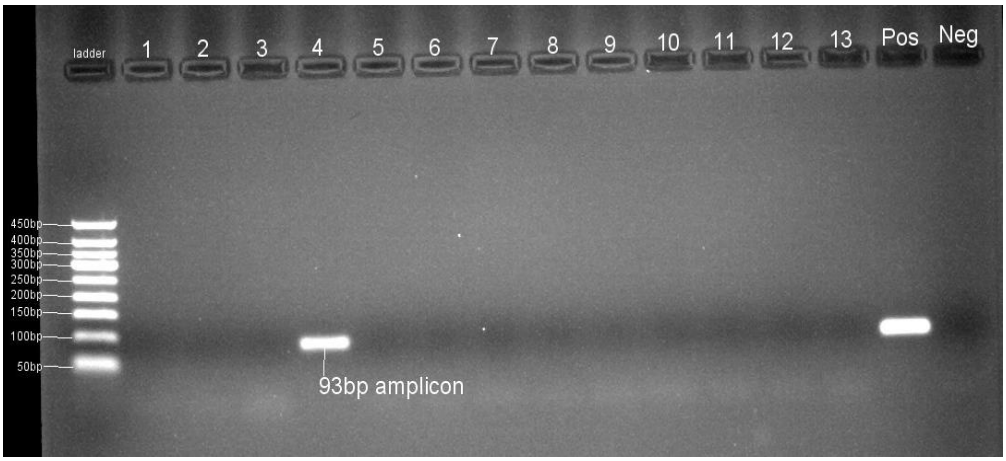
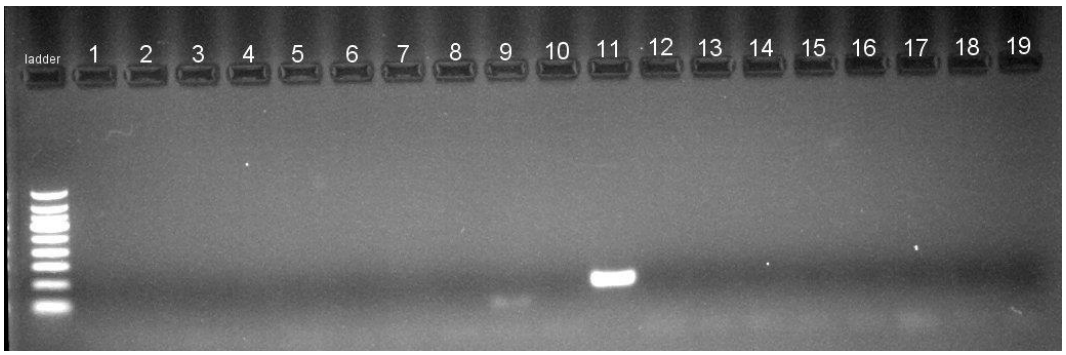


Figure 17: PCR showing positive amplification of 93 bp fragments specific for *exfa* gene of *S. aureus.* Lines: 'ladder', DNA molecular weight marker 50bp; 4, positive isolate; 1 to 3 and 5 to 13: negative isolates; 'pos': positive control; 'neg': negative control.

Following images show amplification of *exfa* gene (93 bp amplicon) in remaining clinical samples with a 50 bp DNA molecular weight marker.



SECTION FOUR: DISCUSSION

S. aureus is a major human microbiota inhabitant as well as a potent pathogen capable of causing a wide range of diseases (Tong et al., 2015). In hospital settings, *S. aureus*, including multi-drug resistant strains such as MRSA, is one of the most common cause of hospital-acquired infections (Shuguang Li et al., 2016; M. Wang et al., 2019); thus treatment is still challenging. Moreover, the bacterium is endowed with a various set of toxins and virulence determinants which elevate its pathogenicity (Tam & Torres, 2019).

In this study, patients' demographic data such as age, gender and relevant clinical information such as hospital service and sample source, were collected from the hospital medical record database. Male patients were overall the most affected subgroup. They represented the highest number of cases with 57.89% (n=44); and age group of 61 to 70 years had the highest number of male patients (n=14; 18.42%). MRSA infections were found to be occurring less frequently in the age group 60 years and below. With regard to the distribution of MRSA infections among patients, the data analysis showed a threefold increase in occurrence of MRSA infections among inpatient subgroup (irrespective to the gender), in comparison to outpatient subgroup; indeed, seventy five percent (n=57) of MRSA infections occurred in inpatients, versus only twenty five percent (n=19) in outpatients. The median age within this group was 66 year. Similar pattern have been described in a recent study where inpatients subgroup accounted for the half of MRSA acquisition (Auguet et al., 2018). Consistent with the results Rahimi & Shokoohizadeh reported in their study in 2016, which focused on characterization of MRSA among inpatients and outpatients, our study found similar patterns regarding the distribution among patients with respect to gender and inpatients/outpatients subgroup (Rahimi & Shokoohizadeh, 2016).

Inpatient care status is correlated with hospital length of stay, thus prolonged exposure to medical procedures such as antibiotic therapy, invasive procedures and devices, increased likelihood to develop a hospital-acquired infection, particularly MRSA infection and augmented cost (Hutzschenreuter et al., 2018). Lakhundi & Zhang, reported

old age, long hospital stay, invasive procedures and colonization stauts as risk factors for MRSA infection (Lakhundi & Zhang, 2018). The first three characteristics are shared within our inpatient group, however, regarding colonization status which has not been investigated in this study, distinguishing whether an infection is caused by a commensal strain or by a hospital-acquired strain is therefore complex. In the same regards, MRSA colonized or infected patients are reservoirs for subsequent transmission within health care facilities (Chow et al., 2017). Standard prevention of transmission procedures should be applied such as systematic screening of MRSA upon patient admission in a hospital, since in most of the cases, infecting strains genotypes were found to match commensal strains one in patients (Lakhundi & Zhang, 2018).

Our study reported a predominance of wound-abscess (n=19; 25%) samples derived from skin foci. This may be due to the fact that primary exposure of *S. aureus* to host tissue is made through breach into skin and/or mucosal surface. One study reported *S. aureus*, mostly MRSA, to be responsible for more than half of investigated skin infections and identified the presence of abscess as a risk factor for MRSA (Valderrama-Beltrán et al., 2019). *S. aureus* is the major cause of SSSI (Esposito et al., 2016; Tong et al., 2015). Establishment of *S. aureus* skin infection provides a portal of entry for the bacteria and is further followed by invasive spread into blood, resulting in bacteremia (Horino & Hori, 2020). Blood samples collected in this study accounted for the second most predominant source which yielded MRSA, with 22.4% (n=17). The highest number of cases (70.5%; 12 of 17) of MRSA bacteremia was reported among patients of 61 year and above. A similar prevalence was reported for the same age group in previous studies (Jayaweera et al., 2017; Jokinen et al., 2018). Such occurrence of MRSA bacteremia in elderly patients is alarming. Bacteremia in elderly patients is associated with high mortality especially in patients with comorbidity factors including malignant diseases and cardiovascular conditions, as well as increased likelihood to develop complications (Dayan et al., 2016; Horino & Hori, 2020). A timely identification of causative strains and source of infection as well as a comprehensive antibiotic resistance profiling are mandatory to guide proper antibiotic therapy. MRSA infections were predominantly found in the cardiology department (n=14; 18.4%) and pulmonary infections department (n=10; 13.2%)

Investigation of molecular determinants in general, and more particularly, characterization of virulence factors is an important field of research concerning *S. aureus* and its related infections. Moreover, the potential extent of a condition is likely dependent on which toxins and associated virulence factors are involved in the pathogenesis. *S. aureus* produces a well-characterized set of toxins which can be classified into three groups based on their functions, they are namely: (i) cytotoxins, (ii) staphylococcal superantigens, (iii) cytotoxic enzymes (Tam & Torres, 2019). This study was focused on two members of staphylococcal toxins, one from the cytotoxins group: α-hemolysin; the other from the cytotoxin enzymes group: exfoliative toxin A. Prevalence of toxin gene carriage in MRSA isolates was investigated using molecular techniques.

Several inflammatory infections are caused by damage to skin epithelium. These damage may be either the result of dermonecrotic activity exhibited by cytotoxins, or desquamative action of exfoliative toxins (Berube & Wardenburg, 2013; Mariutti et al., 2017). Seventy-four (97.36%) of the isolates in this study were found to be positive for *hla* gene. The gene is encoded in the core genome; it must therefore be, as demonstrated by several studies, highly conserved within the species and carried by the large majority of strains (Romaniszyn et al., 2015; Tabor et al., 2016; Xie et al., 2016, 2018). α-hemolysin is a potent pore forming toxin involved in several mechanism; and it has been implicated in the severity of SSSIs, sepsis, multi organ toxicity as well as wide range cell-specific toxicity (Powers et al., 2015; Surewaard et al., 2018). α-hemolysin has been described to impair wound healing in animal models as well as to promote *S. aureus* internalization into mast cells thereby subverting a major component of both innate and adaptive immunity (Goldmann et al., 2016; Putra et al., 2019). Jenkins et al., in 2015, investigated whether transition from commensal *S. aureus* to pathogen was consistent with specific virulence gene upregulation. It has been proven in experimental models that expression of *hla* is upregulated during infective episode with invasive feature, suggesting the role of this toxin in the course of infections (Jenkins et al., 2015). Furthermore, both Putra et al. and Jenkins et al. correlated the role of α-hemolysin in pathogenesis of infection by using mutant strains. They demonstrated reduced virulence attributable to the lack of toxin in mutant strains, compared to wild type strains. In a study examining the contribution of virulence determinants during skin infection model in mouse, using ST93 CA-MRSA and comparing to other clones, Chua et al. reported that hypervirulence

observed in ST93 CA-MRSA clone was most likely due to increased expression of α-hemolysin (Chua et al., 2014). Interestingly, the presence of a given gene does not always correlate with gene integrity or expression of a functional protein, which also applied for α-hemolysin. After sequence typing of *hla* gene, a study reported non- expression by a variant of the toxin due to a stop codon mutation (Sharma-Kuinkel et al., 2015).

For the prevention of *S. aureus* infections or at least, reduction of their severity, α-hemolysin has been regarded as the reasonable target. Pharmacological strategies targeting the β-PFT have been developed (Escajadillo & Nizet, 2018). In experimental models, passive immunization with monoclonal antibodies have yielded satisfactory results. The prophylactic approach used in a recent study with anti-α-hemolysin antibody MEDI 4893 monoclonal antibody showed significant reduction of toxin-mediated mortality in treated mice (Surewaard et al., 2018).

Conversely to α-hemolysin which cannot be directly associated with a particular clinical entity, exfoliative toxin A has been strongly characterized in patients with SSSS. Only two (2.63%) of the MRSA isolates tested in the current study have been found positive to exfoliative toxin A. One of the positive isolates has been recovered from nasal swabs in a 55 years male patient; the second isolate was recovered from wound-abscess of a 2 years female in plastic surgery department. Due to the severe infectious features displayed by *S. aureus* producing ETA, one would not expect to find such virulent strains in high prevalence. In a recent study, ETA gene was found to be present in approximately 1.5% of *S. aureus* strains (Bukowski et al., 2018). Our results are consistent with the one reported by several other studies. The investigation of ETA prevalence made by multiple studies has yielded very low or null results (Shipeng Li et al., 2014; Xie et al., 2016, 2018). However more recently, X. Li et al. and Salas et al. reported significantly high carriage of *eta* gene of 57.3% in China and 73.13% in Spain respectively (X. Li et al., 2019; Salas et al., 2020). A comparison of study relatedness between this current study and the study of Salas et al. in 2020, showing similar sample size and isolate characteristics, could suggest that *eta* gene distribution is correlated with specific geographic location and clonal lineage (X. Wang et al., 2016).

Although ideas hypothesized initially have yielded useful information about the toxin gene carriage pattern, this work suffers from a number of limitations notably related

to the study design. In fact, it is a single center study which focused on 74 clinical isolates; therefore the results above-presented may only reflect MRSA dynamics in the experimented hospital and may not be generalizable to other hospital settings in the country. Multicenter studies involving larger sample size and targeting all classes of *S. aureus* virulence determinants are therefore mandatory. Furthermore, this work has also been limited by the lack of susceptibility testing to other antibiotics. The increasing trend of multi drug resistant (MDR) MRSA renders difficult any attempt of treatment; guiding proper antibiotic therapy may help to reduce length of hospitalization as well as cost of care. Regarding molecular epidemiology, the sole phenotype-based identification of MRSA using cefoxitin disk diffusion, relevant for the study design but yielded limited information about genotypic mechanisms driving such resistance. Future studies that focus on *mecA* gene and its homologous genes (*mecB, mecD, mecC*) as well as *SCCmec* typing, *S. aureus*-specific *spa* typing and sequence typing could sure help investigate genetic characteristics of disease-causing strains.

SECTION FIVE: CONCLUSION

S. aureus represents a major threat to global public health, both in high- and low-income countries. The emergence of various resistance traits and the rising incidence of unusually severe infections further complicate the challenge. In this study, molecular biology techniques were used to characterize toxin carriage patterns in MRSA clinical isolates obtained from patients over a period of 8 years at Near East University Hospital. The outcome of the experimental investigation has led to the conclusion that MRSA strains circulation in the hospital setting carry both *hla* and *eta* genes. Besides, study results indicated that inpatients were likely to be the most affected subgroup, prompting the development of an effective surveillance strategy.

Future investigations are necessary to validate the conclusions presented in this study. Researchers should therefore seek to address this issue in order to develop better understanding of the virulence patterns of *S. aureus* strains present in TRNC. The use of whole genome sequencing, for inter-strains genome comparison purpose as well as the toxin gene and antibiotic resistance pattern screening are among others areas that are yet to be explored.

Overall, this work represents the first study investigating hemolytic and epidermolytic toxin carriage and is a step further towards a more profound understanding of molecular characteristics associated with *S. aureus* strains circulating in hospitals in TRNC. As a result, this study can help develop better strategies, for not only treatment, but also for the prevention and management of infections.

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