TURKISH REPUBLIC OF NORTHERN CYPRUS

NEAR EAST UNIVERSITY

HEALTH SCIENCES INSTITUTE

PREVALENCE OF PANTON-VALENTINE LEUKOCIDIN (PVL) IN METHICILLIN-RESISTANT S*TAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL

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MASTER OF SCIENCE THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY DEPARTMENT

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DECLARATION

I hereby declare that the work in this thesis entitled "**PREVALENCE OF PANTON-VALENTINE LEUKOCIDIN (PVL) IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL**" is the product of my own research efforts undertaken under the supervision of Dr. 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

Danyar Hameed M. Amin. Prevalence of Panton-Valentine Leukocidin (PVL) 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, 2020

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of healthcare associated infections. Panton-Valentine leukocidin (PVL) is a virulence factor, a cytotoxin, produced by some *S. aureus* strains that induces leukocyte lysis and tissue necrosis. PVL-associated *S. aureus* (PVL-SA) predominantly causes skin and soft-tissue infections (SSTIs) but can also lead to life-threatening invasive infections. Although PVL-SA is commonly observed in community-associated methicillin-resistant *S. aureus* (CA-MRSA), reports indicate that PVL-SA in the hospital setting pose an important public health risk. There are no reports on the molecular detection of virulence characteristics or their prevalence of PVL-producing MRSA isolates in Northern Cyprus in literature. The purpose of this study was to determine the prevalence of PVL in MRSA in clinical isolates from patients admitted to a tertiary hospital in Cyprus. Fifty *S. aureus* clinical isolates were obtained from various sites of patients admitted to Near East University Hospital, Northern Cyprus. BD Phoenix automated identification system was used for bacterial identification and antibiotic susceptibility testing. Methicillin resistance was confirmed by disc diffusion assay according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Presence of *nuc* and *mecA* genes was tested by multiplex PCR. Detection of *pvl* gene was performed by single target PCR. Out of 50 *S. aureus* isolates identified as MRSA by BD Phoenix system, 3 isolates were susceptible to cefoxitin with disc diffusion assay. Among 50 isolates, 100 % (50/50) were *nuc* positive and among the *nuc* positive isolates, 68% (34/50) were *mecA* positive. Among 47 confirmed MRSA isolates, 27.7% (13/47) were *pvl* positive. This represents the first study of PVL expression among MRSA isolates in Cyprus. Prevalence of PVL among clinical MRSA isolates in in Near East University Hospital in Northern Cyprus was 27.7%. Reporting of PVL-positive MRSA is central to the monitoring of their clinical impact in patients and guide prevention strategies.

Key words*:* methicillin-resistant *Staphylococcus aureus* (MRSA), Panton-Valentine leucocidin (PVL), infection, virulence, PCR

ÖZET

Danyar Hameed M. Amin. Yakın Doğu Üniversitesi Hastanesi'nde Metisilin Dirençli *Staphylococcus aureus* Klinik İzolatlarında Panton-Valentine Lökosidin (PVL) Prevalansı. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Programı, Yüksek Lisans Tezi, Lefkoşa, 2020

Metisilin dirençli *Staphylococcus aureus* (MRSA) sağlık hizmeti ile ilişkili enfeksiyonların önde gelen nedenidir. Panton-Valentine lökosidin (PVL), lökosit lizisini ve doku nekrozunu indükleyen ve bazı *S. aureus* suşları tarafından üretilen bir sitotoksin ve virülans faktörüdür. PVL ile ilişkili *S. aureus* (PVL-SA) ağırlıklı olarak cilt ve yumuşak doku enfeksiyonlarına neden olmakla birlikte hayatı tehdit eden invaziv enfeksiyonlara da yol açabilir. Her ne kadar PVL-SA, toplum kökenli metisilin dirençli *S. aureus* (CA-MRSA) suşlarında yaygın olarak gözlense de, raporlar hastane ortamındaki PVL-SA'nın önemli bir halk sağlığı riski oluşturduğunu göstermektedir. Kuzey Kıbrıs'taki MRSA izolatlarının virülans özellikleri ve moleküler tespiti, veya suşlarınn PVL sekresyon prevalansı hakkında literatürde herhangi bir rapor bulunmamaktadır. Bu çalışmanın amacı, Kıbrıs'ta bir özel bir hastaneye başvuran hastalardan izole edilen klinik MRSA izolatlarındaki PVL prevalansını belirlemektir. Kuzey Kıbrıs Yakın Doğu Üniversitesi Hastanesi'ne kabul edilen hastalardan elde edilen 50 klinik *S. aureus* izolatı bu çalışmaya dahil edilmiştir. Bakteriyel tanımlama ve antibiyotik duyarlılık testi için BD Phoenix otomatik tanımlama sistemi kullanılmıştır. Metisilin direnci, Avrupa Antimikrobiyal Duyarlılık Testi Komitesi (EUCAST) kriterlerine göre disk difüzyon testi ile doğrulanmıştır. *nuc* ve *mecA* genlerinin varlığı multipleks PZR yöntemi ile test edilmiştir. PVL geninin saptanması tek hedefli PZR ile gerçekleştirilmiştir. BD Phoenix sistemi tarafından MRSA olarak tanımlanan 50 *S. aureus* izolatından 3 izolat, disk difüzyon analizi ile sefoksitine duyarlı olarak tespit edilmiştir. 50 izolatın %100'ü (50/50) *nuc* pozitif olarak saptanmış ve *nuc* pozitif izolatlar arasında %68'i (34/50) *mecA* pozitif olarak belirlenmiştir. Disk difüzyon testi ile konfirme edilmiş 47 MRSA izolatı arasında %27.7 (13/47) *pvl* pozitifliği saptanmıştır. Bu çalışma, Kıbrıs'taki MRSA izolatlarında PVL ekspresyonu rapor eden ilk çalışmadır. Kıbrıs Kıbrıs Yakın Doğu Üniversitesi Hastanesi'nde klinik MRSA izolatlarında PVL prevalansı %27.7 olarak belirlenmiştir. PVL-pozitif MRSA enfeksiyonlarının raporlanması, virülant fenotiplerin hastalar üzerindeki klinik etkilerinin izlenmesinde ve enfeksiyon önleme stratejilerinin geliştirilmesinde merkezi önem taşımaktadır.

Anahtar kelimeler: Metisilin dirençli *Staphylococcus* aureus, Panton-Valentine lökosidin (PVL), enfeksiyon, virülans, PZR

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LIST OF ABBREVIATIONS

PVL	Panton-Valentine leukocidin
DTA	Deep tracheal aspirate
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
PBP2A	Penicillin-binding protein 2 A
CA-MRSA	Community-acquired methicillin resistant *Staphylococcus aureus*
WTA	Wall teichoic acid
TA	Teichoic acid
LTA	Lipoteichoic acid
ECM	Extracellular matrix
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
FnbP	Fibronectin binding protein
CRF	Coagulase reacting factor
ETA	Exfoliative toxin A
ETB	Exfoliative toxin B
TSST-1	Toxic shock syndrome toxin
IE	Infective endocarditis
TNFR-1	Tumor necrosis factor 1 receptor
VRSA	Vancomycin resistant *Staphylococcus aureus*
VISA	Vancomycin intermediate *Staphylococcus aureus*
IVDU	Intravenous drug users
TBE	Tris/Borate/EDTA
SAg	Superantigens
MSA	Mannitol Salt Agar
HVR	Hypervariable area
PBS	Phosphate buffered saline
MHA	Muller-Hinton agar
EUCAST	European Committee on Antimicrobial Susceptibility Testing
PC	Positive Control
NC	Negative Control

SECTION ONE: INTRODUCTION

1.1 Aims and Scope

Staphylococcus aureus is one of the most commonly occurring and significant human pathogens in health care facilities and in the community, and is known to cause a range of diseases including infections of the skin and soft tissue, pneumonia, endocarditis and more invasive infections, including bacteremia and sepsis. It is a significant cause of surgical injuries and diseases associated with medical equipment in hospitals. Although frequently found as part of the human normal flora, *S. aureus* is also responsible for opportunistic infections in the presence of underlying factors such as immune system dysfunction, foreign body invasion and impaired skin integrity as well as infection with another pathogenic agents. It is reported that *S. aureus* colonizes around 30% of the human population (Tong, et al., 2015).

A rapid increase in *S. aureus* antibiotic resistance rates has been observed in recent years, which has been linked with high mortality and morbidity in patients. The ability of *S. aureus* to develop resistance to all antibiotics used in treatment, particularly to methicillin has become a major problem in the hospital setting and represents a global threat to human health. Methicillin resistance occurs via the synthesis of a new penicillin-binding protein (PBP2A), under the control of *mecA* and less often of *mecC*, which is the target region of antibiotics with a beta-lactam ring. Consequently, methicillin resistance

induces resistance to all beta-lactam antibiotics except some cephalosporins. Methicillin resistant *S. aureus* (MRSA) was initially detected in 1961 and remains a serious health problem today.

Almost all strains of *S. aureus* have invasive and virulent characteristics. They independently produce a group of enzymes, including nucleases, proteases, lipases, hyaluronidases, collagenases and thermostable nucleases as well as *staphylococcal* protein A (encoded in the *spa* gene) which help bacteria propagate into human tissues, inhibit the host immune response and transform host-tissues into nutrients required for the bacterium to survive and disseminate within the human host (Everitt, et al., 2014). Nucleases, encoded by *nuc* gene, are present in all *S. aureus* strains and can be used to distinguish *S. aureus* from coagulase-negative *staphylococci* (Brakstad, et al., 1992).

S. aureus pathogenicity relates to several virulence factors which allow a body to respond, to escape from immune system and cause harm to the host. Among a wide range of virulence factors that facilitate the establishment of infections in the human host, leukotoxins, including Panton-Valentine Leukocidin (PVL) stand out particularly in community-acquired skin and soft tissue infections (Spaan, et al., 2014). Sir Philip Noel Panton and Francis Valentine named the Panton-Valentine Leukocidin in 1932 which was associated with soft tissues infections (Prevost, et al., 1995). PVL, encoded by both co-transcribed genes, *lukS-PV* and *lukF-PV*, is a major cytotoxin produced by certain strains of *S. aureus* (Genestier, et al., 2005). It is frequently detected in isolates from human abscesses, furuncles and severe necrotic pneumonia acquired in the community (Lina, et al., 1999; Cribier, et al., 1992). PVL induces leukocytosis and tissue necrosis by means of

pore-formation in the target cells, exerting its toxic effects a result of the synergistic performance of two separate proteins (LUK S-PV and LUK F-PV) (Finck-Barbancison , et al., 1993). PVL producing MRSA strains have been reported in mild skin and soft tissue infections, however severe pneumonia and sepsis cases of MRSA have also been observed (Maltezou, et al., 2006). A clear association between severe pneumonia and *S. aureus* strains containing the PVL gene was demonstrated in several clinical studies (Vandenesch, et al., 2003). PVL is found in the majority of MRSA isolates within the population and rarely found in hospitals and clinics, and therefore it is considered as a predictor of community-acquired MRSA (CA-MRSA) infection. However, although CA-MRSA strains are more likely to produce PVL, some recent studies have shown that the transmission of PVL-containing *S. aureus* isolates from the community to the hospital setting (Dharm, et al., 2016). This represents a remarkable risk to public health.

In Northern Cyprus, there are no molecular surveillance data for *S. aureus* strains, particularly for MRSA strains, isolated from hospitals or the community. Therefore, MRSA strains remain largely uncharacterized in terms of their virulence characteristics. Molecular detection of antibiotic resistance and molecular markers is vital for understanding the pathogenicity of strains circulating in hospitals and can help healthcare professionals identify transmission routes as well as the best possible treatment options for the patients. The aim of this study was to detect methicillin resistance in *S. aureus* strains isolated from patients admitted to Near East University Hospital in Northern Cyprus using molecular methods and investigate the prevalence of PVL-containing strains in the hospital setting for the first time.

2. General Information

2.1 Classification

In its formulation of the II version of Bergey's Manual for Systematic Bacteriology, the *Staphylococcaceae* genus was first suggested in a taxonomic manner. The Staphylococcal family comprises the genera *Jeotgalicoccus, Macrococcus, Nosocomiicoccal* and *Salinicoccus*, in addition to the staphylococcal group. From different samples of food and environmental, *Jeotgalicoccus* and *Salinicoccus* species were recovered. Isolation of saline bottle surfaces used in wound purification has been recorded for *Nosocomiicoccus* ampullae. To date, seven species adapted to hoofed animals exist in the genus of *Macrococcus* (Alves, et al., 2008).

Staphylococcus was initially believed to belong to the family *Micrococcaceae*, however later molecular and phylogenetic analysis revealed that staphylococci are not closely related to *Micrococci* anymore, and are thus classified in a new family, named *Staphylococcaceae*.

Although the genera *Staphylococcus* and *Micrococcus* have also been classed in the same family with the genera *Planococcus* and *Stomatococcus*, the numerous gram-positive, catalase-positive cocci have shown no strong ties in named *Micrococcaceae*. Currently *Bacillales* of the genus Bacilli belongs to the order Staphylococcaceae family along with *Bacillaceae, Listeriaceae*, *Paenibacillaceae, Planococcaceae* and other groups. The bacilli belong to the Phylum *Firmicutes*, comprising of Gram positive bacteria

with a fairly small G+C concentration of DNA. In comparison, phylum Actinobacteria now possess strong DNA G+C content of micrococcus organisms. Many of the micrococci is reclassified and reorganized into two families: the redefined *Micrococcaceae* family and the newly formed Dermacoccaceae family. Both are from the *Micrococcineae* (*Actinobacteria* class) suborder (Stackebrandt, et al., 2000).

As of 2014, the genus *Staphylococcus* genus was validly described to consist of 47 species and 23 sub species. Regularly *Staphylococci* associated with human infection are *S. aureus*, *S. epidermidis* and *S. saprophyticus*.

2.2 *Staphylococcus aureus*

S. aureus is amongst the most polyvalent organisms in the world of microscopy. It is typically found in the skin and body portals such as nasal passageways, eyes and ears as temporary colonizers (Figure 1) and around 20-30% of human beings are asymptomatically colonized. As a member of the normal flora, *S. aureus* can also become an opportunistic pathogen leading to a wide range of potential infections.

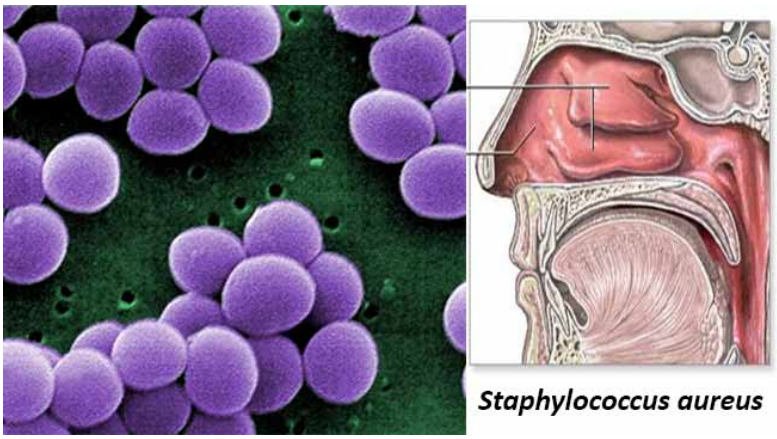

Figure 1: Colonization of human mucous membranes by *S. aureus*. (Mulcahy et al., 2012; Baur et al., 2014; Burian et al., 2010).

Nevertheless, any skin trauma in people with compromised immune systems can offer these bacteria the opportunity to cause infection. There are two possible mechanisms can mediate the disease process:

1) production of toxins, and

2) colonization on the host cell which causes invasion and destruction of tissues

Owing to its multiple virulence factors, *S. aureus* is well suited with antimicrobial tolerance mechanisms to cause severe infections. *S. aureus* is frequently present in many environments and can survive long periods on dry surfaces. Bacteria are resistant to elevated temperatures, antiseptics and disinfectants. It is now recognized that, due to inadequate hand washing, *S. aureus* is the most frequently transmitted bacterium among health care staff (Carboneau, et al., 2010).

2.2.1 Morphology and Species Properties

S. aureus is a facultative anaerobic Gram-positive coccus having a diameter of 1μm - 1.3μm. On microscopic examination, the organisms appear in singly, in pairs, and irregular clusters, as bunches of grapes, they are nonmotile, non-spore forming, catalase and coagulase positive. Typical colonies are yellow to golden yellow in color, smooth, whole and slightly raised on 5% sheep blood agar. Many strains appear as nonhemolytic. It also induces successful deoxyribonuclease and mannitol fermentation. Some strains express toxins which target the gastrointestinal tract. *S. aureus* enterotoxins are heat-stable, resistant to 30-70 minutes heating at 100°C (Stegger, et al., 2014).

2.2.2. Structural Components

Due to the plethora of identified virulence factors *S. aureus* is known to be one of the most pathogenic bacterial microorganisms.

2.2.2.1 Microcapsule

Capsule is a large polysaccharide structure which lies outside the bacterial cell membrane. Such polymers are made up of two to four monosaccharide replicated oligosaccharide groups. Surface-associated, reduced in anti-genetic sensitivity and strongly conserved in clinical isolates are *S. aureus* capsular antigens. Capsule prevents phagocytosis, facilitates the organism's evasion from the host immune system. Most *staphylococci* are

microcapsule producers. Types 5 and 8 constitute 75% of human infections in the 11 forms of micro capsular polysaccharides that were reported. Type 5 has been commonly found in MRSA strains. Four of these anti-phagocytic polysaccharides, including forms 5 and 8, have been statistically determined to have a statistically related structure (Katherine, et al., 2004).

2.2.2.2 Cell Wall

The *staphylococcal* cell wall has a weight of 50% peptidoglycan. Peptidoglycan is made up of alternative N-acetylglucosamine polysaccharide and 1,4-b associated N-acetylmuramic acid. The peptidoglycan chains are linked to N-acetylmuramic acid by tetrapeptide chains and *S. aureus*-specific pentaglycine bridge. Peptidoglycan can have endotoxin-like activity to promote cytokine release by macrophage, complementary activation and platelet aggregation. Differences in *staphylococcal* peptidoglycan structure can lead to changes in the efficiency of disseminated intravascular coagulation. The main components of *S. aureus* cell wall are teichoic acid (TA). TA are of two separate types: wall teichoic acids (WTA). and lipoteichoic acid (LTA). The cell wall is covalently linked with peptidoglycan in the bacterial cell wall, and the cell wall is anchored in the cytoplasmic membrane. TA overexpression improves the *S. aureus* virulence by aiding adhesion to abiotic surfaces. Furthermore, modification of TAs in D-alanine (D-Ala) lead to susceptibility to antimicrobial cationic peptides such as defenders or cathelicidins and

to antibiotics with glycopeptides such as vancomycin or teicoplanin (Speziale, et al., 2009).

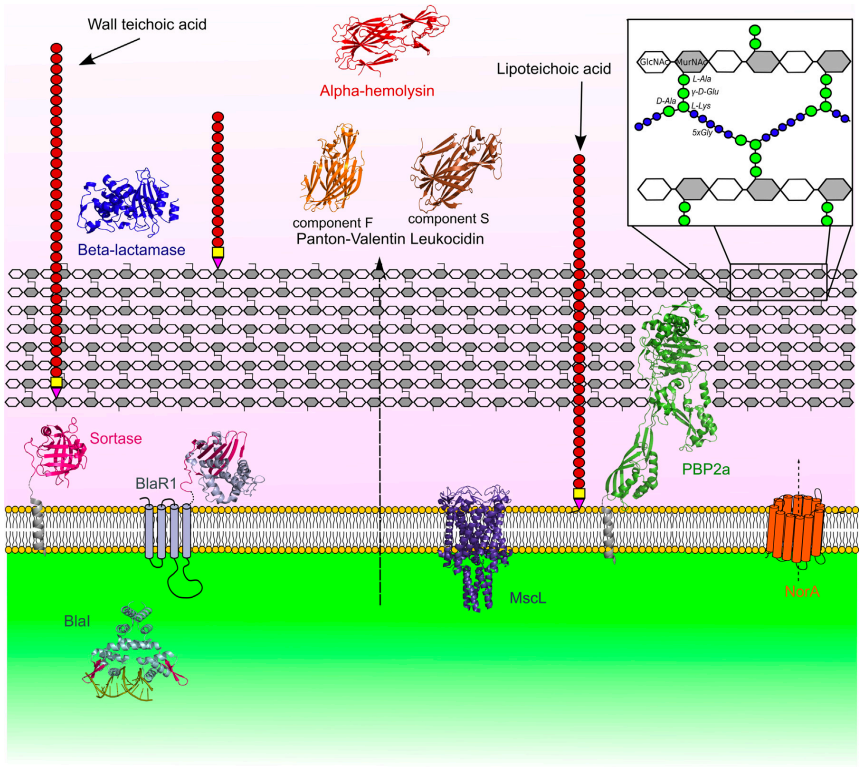

Figure 2: Diagram of characteristics of the *S. aureus* cell wall. (Assis, et al., 2017)

2.2.2.3 Cell Wall Components

ECM, an extracellular portion of animal tissue, provides a structural frame for human tissues. Components of the ECM are intracellularly generated and discharged into the extracellular atmosphere by resident cells (Beckerle & Mary, 2001). Collagen, non-collagen glycoproteins, and proteoglycans are major components found in ECM. The redundancy of genes which encode isoforms of the same molecule (i.e. collagen) and

differential splicing generates a great diversity in the glycoproteins Fn and thrombospondin. Many ECM glycoproteins are large molecules which stretch for many hundred nanometers in conformation. Many pathogenic agents, such as *S. aureus*, have been shown to use ECM to bind to and colonize the host-tissues by specific bacterial cell-surface MSCRAMMs (Rivas, et al., 2004; Rivera, et al., 2007; Speziale, et al., 2009),

A broad variety of surface-related factors mediating bacterial relations to the substratum, adhesins, are used by *S. aureus*. A major class of *S. aureus* adhesives includes proteins anchored covalently to cell peptidoglycans which specifically bind to plasma or host ECM components and are collectively called the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Figure 3). Such molecules identify the most influential host ECM components or blood plasma elements such as fibronectin (Fn) and collagen and fibrinogen (Fbg) (Patti, et al., 1994).

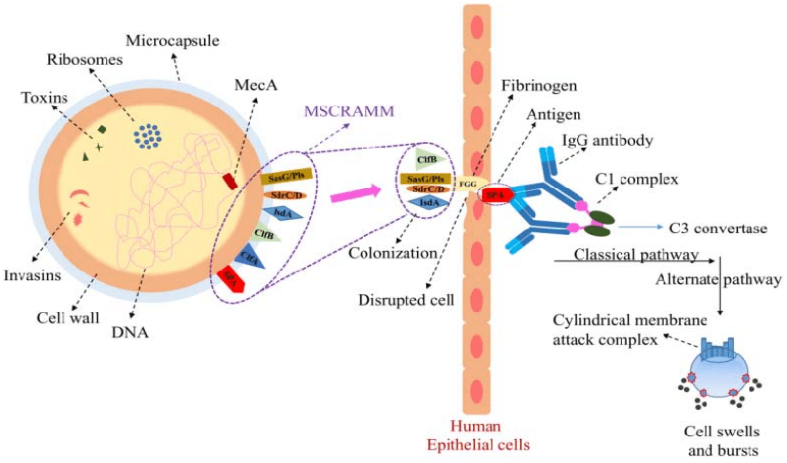

Figure 3: *S. aureus* Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) (Berkley, et al., 2005).

2.2.3 Virulence Factors

2.2.3.1 Surface Proteins

There are many structural features of several *staphylococcal* surface proteins (Figure 4). Such characteristics include a series of a coded signal at the N terminal, positive-loaded amino acids that extend through the cytoplasm, a membrane-span-hydrophobic domain and a cell wall-anchor area at the carboxy terminal. A N terminal ligand-binding area that is revealed on the surface of the bacterial cell may serve as adhesives for some of these proteins (Foster, et al., 1994). The prototype of these proteins is protein A. As a virulence factor, protein A displays multi-faceted functions. It possesses antiphagocytic properties based on its ability to bind the human immunoglobulin Fc portion. Protein A attaches to the Fc component of the immunoglobulin, and protects *S. aureus* from opsono-phagocytosis (getting engulfed and then destruction by the immune cells). Protein A biofilm production allows attachment of *S. aureus* to covered surfaces such as endovascular catheters, coated by von Willebrand factor (VWF). Protein A is often known to cause inflammation in the lung by attaching to a tumor necrosis factor 1 receptor (TNFR-1) broadly distributed on the epithelial of the airways. This relationship plays a crucial role in *staphylococcal* pneumonia pathogenesis (Katherine, et al., 2004). Additionally, *S. aureus* has the ability of biofilm production by clinically important MRSA, facilitated by fibronectin-binding protein A (FnBPA) and fibronectin-binding protein B (FnBPB). Collagen-binding protein is also one of *S. aureus* cell surface adhesion proteins, which is essential for bacterial-host adhesion and for immune evasion. Any of

these associated proteins bind extracellular molecules and Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). These proteins play an important role in colonizing the host tissue as described in recent studies (Patti, et al., 1994).

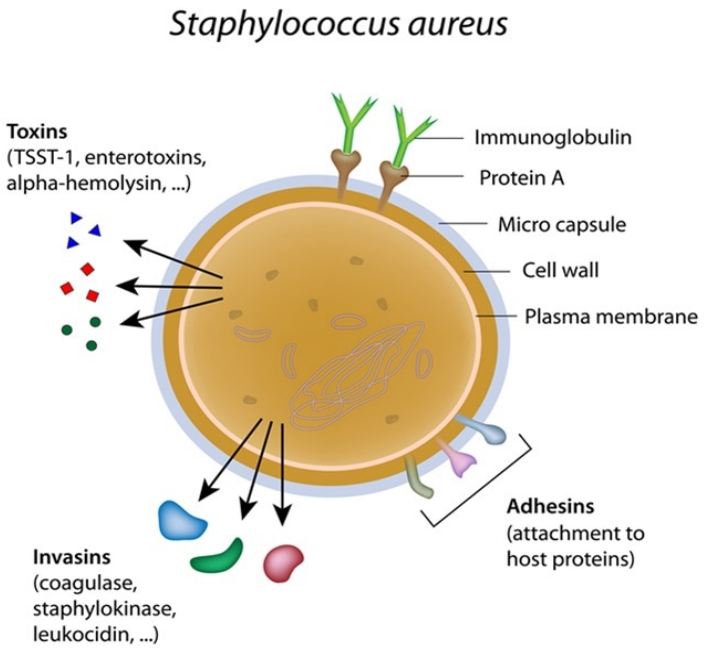

Figure 4: *S. aureus* have surface proteins that are more often present on epithelial and endothelial surfaces to facilitate adhesion to host proteins. Many strains have a clumping component. *S. aureus* induces host cell adhesion. Fibronectin, fibrinogen-compounding (FnbP) proteins, and bacterial cell surface collagen-rich receptors promote adherence to damaged tissue (Alila, et al., 2017).

2.2.3.2. Enzymes

Staphylococci secrete numerous enzymes that are tissue-destroying, such as protease, lipase and hyaluronidase. Although their role in pathogenesis of the disease is not clearly determined, these bacterial products can facilitate the spread of infection through neighboring tissues.

Coagulase: coagulase (free coagulase) enables the usual coagulase reacting factor (CRF) in plasma, allowing the plasma to coagulate by transforming fibrinogen to fibrin and may serve to cover the bacterial cells with fibrin, rendering them immune to opsonization and phagocytosis.

Staphylokinase (fibrinolysin): staphylokinase has an antigenic and enzymatic fibrin function, and often splits fibrin clot and allows infection spread to neighboring tissues.

Hyaluronidase: degrade hyaluronic acid in the intercellular layer of the connective tissues and allow bacterial spread to the neighboring areas causing damage (Mistretta, et al., 2019).

Deoxyribonuclease: induces DNA degradation

Lipase: degrades lipids

Phospholipases: degrades phospholipases

Proteases: causes protein lysis (Maiques, et al., 2006).

2.2.3.3. Toxins

Staphylococci secrete a significant number of toxins clustered according to their action mechanisms.

Cytolytic toxins: *S. aureus* produces hemolysins, known as alpha (α), beta (β) and delta (δ) toxins, which primarily mediate red blood cell destruction by creation of pores and create proinflammatory modifications in mammalian cells. The subsequent cell damage can lead to sepsis syndrome manifestations. The bacterium also produces leukocidins that target polymorphonuclear leucocytes. Staphylococcal leukocidin is a cytotoxin, β-pore-forming toxin called Panton-Valentine leucocidin (PVL). PVL creates lytic pores in the cell membranes of neutrophils and induces the release of neutrophil chemotactic factors that promote inflammation and tissue destruction. It is linked with skin and soft tissue infections epidemiologically (Sully, et al., 2014).

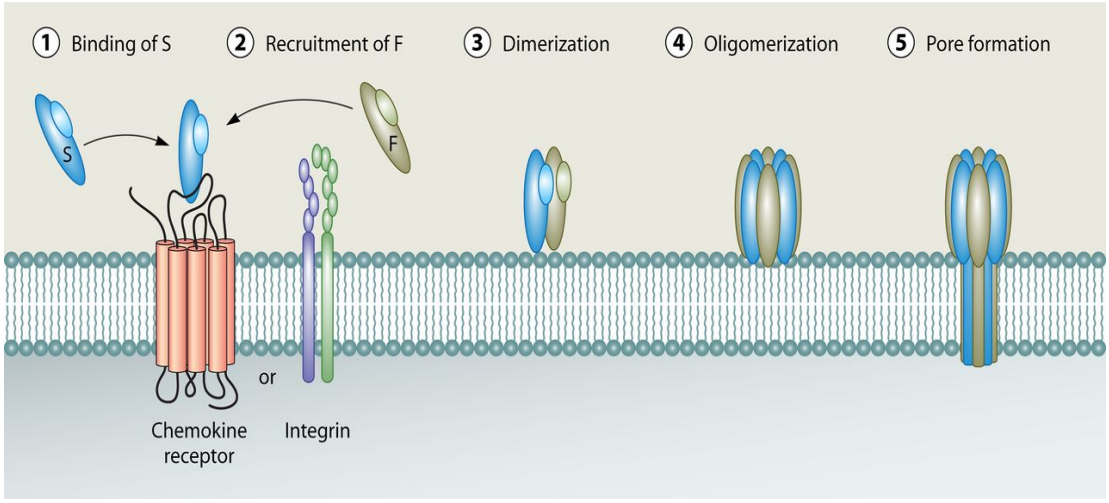

Figure 5: Current model of PVL pore formation in host cells (Alonzo, et al., 2014).

PVL pore formation is believed to occur in a stepwise fashion that begins with toxin recognition of cellular receptors on the surface of target host cells (Figure 5). On most host cells, the "S" subunit recognizes a proteinaceous receptor (either a chemokine receptor [LukED and PVL] or an integrin [LukAB/HG]) to facilitate high-affinity binding to the cell surface. The S subunit then recognizes and recruits the "F" subunit, leading to dimerization on the host cell surface. Dimerization is followed by oligomer formation. Toxin oligomers assemble into an octameric prepore structure containing alternating S and F subunits. Following oligomerization, a major structural change occurs in the stem domains of the S and F subunits, leading to membrane insertion and the formation of a β-barrel pore that spans the host cell lipid bilayer (Alonzo, et al., 2014)**.**

Enterotoxins: *S. aureus* enterotoxins are potent gastrointestinal exotoxins that are involved in food poisoning, toxic shock syndrome and staphylococcal infectious diseases in human. Nine antigenic forms (A-J excluding F) exist. Some strains may contain multiple enterotoxins.

Exfoliative (epidermolytic toxin): Two type of epidermolytic toxins of *S. aureus* exist – exfoliative toxin A (ETA) and B (ETB). These toxins are serine protease that allow desmosomes or intercellular bridges in the granulosum stratum to separate. Staphylococcal skin disease, in which the outer epidermis layer is removed from the underlying tissue, is induced by the epidermolytic toxins (Mistretta, et al., 2019).

Toxic shock syndrome toxin (TSST-1): TSST is a staphylococcal superantigen (SAg). The major functions of TSST-1 are induction of cytokine release from macrophage and T lymphocytes and induction of leakage of endothelial cells. Staphylococcal superantigens penetrate mucosal barrier and are responsible for virtually all menstrual toxic shock syndromes. The two disorders triggered by these toxins, toxic shock syndrome and alimentary poisoning, are responsible for specific fields of the enterotoxin molecule. Although there is little homology in the amino acid sequence, toxic shock toxin 1 is very similar in structure to enterotoxins B and C. 20% of *S. aureus* isolates contain the gene for TSST-1 (Sospedra, et al., 2012; Srevens, et al., 2006).

2.3 Epidemiology of *Staphylococcus aureus*

2.3.1 Colonization and Infection

Human beings are natural repository of *S. aureus*. Thirty percent (30%) of health people are known to be colonized (Sakr, et al., 2018). Methicillin sensitive isolates are chronic colonizers, as are methicillin resistant isolates. The risk of future infections in type 1 diabetes patients with staphylococcal colonization is increased (Tuazon, et al., 1975) as in intravenous drug users (IVDU), consumers with drugs, (Tuazon, et al., 1974) hemodialysis patients (Yu, et al., 1986), patients of surgery, (Bigliani, et al., 1995) and the immunodeficiency disease patients. The correlation between colonization and nasopharyngeal septicemia has also been reported in patients infected with the Human

Immunodeficiency Virus (HIV) (Weinke, et al., 1992). The risk of staphylococcal disease is also increased in patients with qualitative or quantitative leukocytic defects (Waldvogel & Francis, 1995).

2.3.2 Transmission

Individuals that are colonized with *S. aureus* strains are potentially at risk of infection. Most cases of nosocomial infections occur by exposure to medical professionals after staphylococci has been temporarily colonized from their own reservoir or from contact with an infected patient. Exposure to a single long-time carrier can also contribute to outbreaks, although such types of transmission are less popular (Casewell, et al., 1996).

2.3.3 Temporal Trends in *S. aureus* Disease

Over the past 20 years, the amount of staphylococcal infections acquired in the community and hospital has increased. This phenomenon correlates with the growing usage of IV devices (Steinberg, et al., 1996). From 1990 to 1992, *S. aureus* was the most common source for nosocomial pneumonia and surgery-wound infections, according to evidence from the Regional Nosocomial Infections Monitoring Program of the Centers for Disease Control and Prevention, was the second most frequent source after coagulase-negative staphylococci of nosocomial contaminated blood streams (Emori, et al., 1993). A second pattern was the drastic global increase in the proportion of infections induced

by MRSA (Panlilio, et al., 1992). National Nosocomial Infections Surveillance, for the period 1987 to 1997, shows that numbers of methicillin-resistant *S. aureus* infections in intensive care units has increased. *S. aureus* is a serious health issue contributing to hemodialysis patients' hospitalization, morbidity and mortality. Prevalence analysis showed that *S. aureus* colonization of 42% of maintenance hemodialysis patients was identified with lateral, oropharynx and inguinal narrows, with 6% of MRSA patients. Certain patients that were diagnosed with *S. aureus*, and MRSA colonization were 33 per cent increased through external surveillance. This is close to other experiments that investigate the efficacy of extra-nasal *S. aureus* colonization in high-risk individuals (Eells, et al., 2015). Trends in correlation with the colonization of *S. aureus* extra-nasal and young people and the causes of not hospitalized in previous 12 months can be indicators of *S. aureus* extra-nasal colonization in younger and healthy patients with a hemodialysis. In our post-hoc analyzes the relation between the colonization of younger and overall *S. aureus* was important. This relationship is unexpected in an era when *S. aureus*, affiliated with the group, is widespread and prevalent among younger persons (Maree, et al., 2007). The nasal carriages of *S. aureus* are considered to contribute considerably to the morbidity, death, and expense of end-stage renal diseases treatment. as the natural vector for *S. aureus* and MRSA infections (Kallen, et al., 2010). A study of households with a documented experience of the current *S. aureus* skin infection found that up to 50% of household members were colonized with *S. aureus* and 48% of *S. aureus* colonization and 51% of MRSA colonization will be absent in nares-only surveys. The scale of pharyngeal and inguinal colonization has triggered a fundamental shift in the understanding of *S. aureus* body colonization (Miller, et al., 2012; Diep, et al., 2008).

2.4 Staphylococcal Diseases

S. aureus is an infectious organism that colonizes and infects both immunocompromised patients and stable immunocompetent population. The skin and nasopharynx of the human body are naturally colonized by this bacterium. Local infections, most of which are lesser than life threatening such as eyes, urethra, vagina, and gastrointestinal tract infections are caused by *S. aureus*. In the hospital environment, the development of resistant strains of *S. aureus* is commonly observed. *S. aureus* has a range of virulence components related to infection pathogenesis. These have different functions and may function either individually or in concert. The role of these bacterial factors in infection is well established (McCrae, et al., 1997; Projan, et al., 1997). *S. aureus* is a commensal of the scalp, axillas, womb and pharynx (Noble, et al., 1967). Skin breach or mucosal barrier allows staphylococci to penetrate into surrounding tissue or bloodstream. It depends upon a dynamic relationship between *S. aureus* virulence factors and processes of host defense whether an infection is confined or spreads (Casewell, et al., 1986).

S. aureus is a primary cause of skin, soft tissues, digestive, bone, joint and endovascular diseases infections which may be life-threatening. Many of such diseases arise in individuals with numerous infection risk factors (Musher, et al., 1994).

2.4.1 Bacteremia

Bacteremia related to *S. aureus* is a life-threatening, high morbidity and mortality infection, which sometimes contribute to metastatic infections including infectious endocarditis, that have a detrimental impact on patient outcome. Some localized *S. aureus* infections, which induce infections to metastatic foci, may exist, which is most prevalent in intravascular catheters and other foreign bodies. Intravascular catheters and other invasive bodies are frequently infected by *S. epidermidis* and other coagulase-negative staphylococci as they can form biofilms on such products. The main cause of morbidity and mortality in compromised patients, particularly with prolonged staphylococcal bacteremia, is staphylococcal bacteremia (Bush & Perez, 2019).

2.4.2 Endocarditis

S. aureus is a key cause of infectious endocarditis which, despite enhanced diagn osis and treatment techniques, remains high in terms of mortality over time (Guerrero, et al., 2009). For decades, *S. aureus* infective endocarditis (IE) was seen as a disease primarily acquired by the population, especially correlated with injection drug usage. In comparison with *S. aureus*-associated bacteremia, rates of nosocomial or intravascular catheter-IE are lower.

2.4.3 Metastatic Infections

S. aureus can spread to various body sites, including the skin, limbs, knees and kidneys. Collection on these sites serve as potential source of recurring infections. The involvement of suppurative materials collected should be assessed for patients with chronic fever following adequate care (Musher, et al., 1994).

2.4.4 Sepsis

Sepsis leads to a subset of local or bacteremia infections. Immunosuppression, chemotherapy and surgical treatments provide contributing factors for septic disease. *S. aureus* is one of the most prevalent Gram-positive pathogens detected in sepsis cases. Severe cases proceed towards multi-organ failure, intravascular clotting, lactic acidosis, and death (Bone & Roger, 1994).

2.4.5 Toxic Shock Syndrome

Toxic shock syndrome (TSS) is a serious, life-threatening toxin-mediated illness, and is usually precipitated by *S. aureus* infection. High fever, swelling, hypotension, multiorgan dysfunction (including at least two or more organ systems) and desquamation (typically of palms and soils) are defined 1-2 weeks after acute disease exists. Severe myalgia, diarrhea, fatigue and neurological abnormalities can occur as well as clinical

syndrome (Pinsky & Michael, 2018). In 1978, Todd et al., who documented the disease in a group of 7 infants, initially identified *S. aureus* toxic shock syndrome (TSS) (Herzer & Christopher, 2001).

2.5 Laboratory Identification of *Staphylococcus aureus*

2.5.1 Gram Staining

Gram staining can be used to discriminate between Gram positive and Gram-negative bacteria. *S. aureus* are Gram positive cocci with distinctive clusters and cocci can occur independently in a couple or in a short line.

2.5.2 Culture

Blood Agar: Blood agar is used to culture and growth of Gram-positive bacteria such as *S. aureus*. Within 18 to 24 hours, yellow or golden yellow colonies with or without beta-hemolysis can be observed.

Mannitol Salt Agar (MSA): MSA is the most widely used selective medium for isolation of *S. aureus*. MSA plates are incubated for 24 to 48 hours at 35°C after inoculation. *S. aureus* is a bacterium that ferments mannitol and produce colonies of yellow or gold.

2.5.3 Biochemical Tests

Catalase test: It is commonly used to distinguish between staphylococci and streptococci. A positive result will indicate staphylococcus and negative result will be streptococcus.

Coagulase Test: The coagulation method is used in order to differentiate *S. aureus* and other staphylococcal species, mainly coagulase-negative staphylococci *(S. epidermidis, S. saprophyticus).*

2.5.4 Molecular Identification by PCR

Polymerase chain reaction (PCR) method provides a fast and efficient approach to organism recognition. Several researchers have identified various targets for PCR identification of *S. aureus*. A multiplex PCR is commonly used to classify MRSA. The test detects 2 genes; *nuc* gene which codes for a *S. aureus*-specific thermostable nuclease and *mecA* gene which encodes the PBP2a which induces resistance to beta-lactam antibiotics. Studies have identified tests for the detection of *mecA* and *nuc* genes to identify *S. aureus* (Costa, et al., 2005).

2.5.5 Detection of Oxacillin/Methicillin Resistance in *S. aureus*

Methicillin diffusion procedure has been carried out to diagnose methicillin tolerance in *S. aureus*, but was later substituted by oxacillin because the oxacillin is more

stable than methicillin during storage and is more prone to hetero-resistant strains. Recently, cefoxitin has also been used as a replacement in disk diffusion studies as it activates the *mecA* gene. Cefoxitin experiments produce more reliable and accurate outcomes than oxacillin studies. Disc-diffusion assays are performed on Mueller–Hinton agar on which a bacterial suspension of McFarland standard is used to standardize the approximate quantity of bacteria used in the antibiotic susceptibility test (Lee, et al., 2001).

2.6 Treatment of *Staphylococcus aureus* Infections

2.6.1 β-lactam Drugs

Currently about 80% of *S. aureus isolates* are resistant to penicillin (Deurenberg, et al., 2007). Other antimicrobials similar to penicillin, such as methicillin, oxacillin and ampicillin, have also been used. A few years after the methicillin was introduced, the strains of resistant were detected. MRSA strains are known to be resistant to all penicillin drugs including oxacillin. There have been several other penicillin drugs (such as amoxicillin, piperacillin and ticarcillin) developed; some have been applied to *S. aureus* therapy (Goto, et al., 2009).

Compounds with a form of β-lactam, but low antimicrobial activity, have been identified in the 1970s. Such compounds have been shown to be β-lactamase (a

microorganism-produced enzyme to regulate β-lactam) inhibitor not to be effective as antimicrobials themselves. The variation of these three antagonists is either amoxicillin / clavulanic acid, ampicillin / tazobactam / sulbactam and piperacillin. Such medications have increased penicillin capacity to induce microorganism death but have not fully alleviated the question of resistance (Verraes, et al., 2013).

More β-lactam antibiotics were discovered or synthetically produced over the years. There are cephalosporins, carbapenems and monobactam. Over the years, many historical advances have been made in cephalosporins. The first generation cephalosporins had the highest activity against aerobic Gram-positive cocci. The following generations had enhanced activity against the gram negative enterobacteria and anaerobes. In the fourth (cefepime) and fifth generations (ceftobiprole and ceftaroline), greater focus was put on efficacy of positive cocci to combat MRSA (Reygaert & Wanda, 2010). Carbapenem medications are known to have the highest and widest range of action against gram positive and Gram-negative bacteria, and are structurally linked with β-lactamase inhibitor products. Imipenem became the first carbapenem medication on the market. The most common carbapenem drugs include meropenem, doripenem and ertapenem. Like other β-lactam products, antimicrobial susceptibility concerns arose in cephalosporins and carbapenems. Some of such drugs are not recommended to be used in MRSA monotherapy (Maduka-Ezeh, et al., 2011). The monobactams have not done a great deal with gram-positive cocci, since as they do not contain other ring attached to the β-Lactam ring structure (Clark, et al., 2008).

2.6.2 Other Cell Wall Drugs

β-lactam drugs are active in microorganisms against synthesis of the cell wall. Glycopeptides are another category of drugs with action against cell wall synthesis; vancomycin is the principal component. Vancomycin has been deemed the medication to combat such diseases with the rise in antimicrobial-resistant strains of the *S. aureus* (e.g. MRSA). Over the last 8 years, however, vancomycin-resistant *S. aureus* strains have emerged (Sievert, et al., 2008). There is also a lipopeptide antimicrobial group of drugs active in cell membrane depolarization. Daptomycin is currently the only drug of this group on the market. There is no major resistance yet recorded as it has only been on the market since 2003. However, low rates of resistance have been detected. The potential of some *S. aureus* strains to display a decreased immunity to daptomycin during therapy has been observed (Sharma, et al., 2008).

2.6.3 Drugs That Inhibit Protein Synthesis

There are many classes of antimicrobial drugs that prevent the production of protein by connecting to either the 30S or 50S ribosomal subunits in bacteria. They include aminoglycosides, tetracyclines (tetracycline, minocycline, tigecycline), chloramphenicol and lincosamides (clindamycin - mainly used for anaerobic microorganisms); macrolides, (azithromycin, erythromycin, clarithromycin); oxazolidine (linezolid); and streptogramines (quinupristine/dalfopristine), (cyclin, tigecycline), tetracycline and

tetracycline. With any of these product classes, antimicrobial resistance cases have been reported: aminoglycosides (Hamdad, et al., 2006), tetracyclines, lincosamides and chloramphenicol (Gould, et al., 2010) the macrolides (Denton, et al., 2008) and the streptogramins (Adaleti, et al., 2010). The oxazolidinones are a fairly recent category and linezolid has only been on the market since 2000. Some studies of low to moderate resistance however have been released (Sader, et al., 2013).

2.6.4 Drugs That Inhibit Nucleic Acid Synthesis

This category of antimicrobials, known as quinolones, prevent nucleic acid synthesis. Quinolone is the name of the drugs alluded to in their first generation. Fluoroquinolones are the next group. Ciprofloxacin, norfloxacin and ofloxacin are part of the second wave, levofloxacin is the third generation and gatifloxacin and moxifloxacin are used in the fourth group.

2.6.5 Drugs That Are Metabolic Pathway Inhibitors

The use of compounds that suppress microbial metabolic processes is an important antimicrobial process. The most widely used drugs rely on microorganisms' mechanism of folate biosynthesis. Part of this mechanism is blocked by sulfa drugs (sulfonamides). These drugs have a very similar structure to para-aminobenzoic acid (pABA), a substrate

needed in one stage of this process. Sulfate drugs may be inserted in the active position of the enzyme which catalyzes the reaction which prepares pABA for glutamate combination. They block pABA's potential for docking and stopping trajectory progression at that stage. The medication trimethoprim hampers a particular stage in the process. Trimethoprim binds to a particular enzyme and is known to prevent its function. A sulfonamide medication, sulfamethoxazole, and trimethoprim are used as a combined medication in order to guarantee that the receptor is completely blocked. Resistance has been documented (Diekema, et al., 2001; Eliopoulos, et al., 2001).

2.7 Mechanisms of Antibiotic Resistance in *S. aureus* and Methicillin Resistance

The excessive use of antibiotics has led to the emergence of multiple drug resistant *S. aureus* strains (Lowy, 1998). Penicillin was introduced in the treatment of *S. aureus* infections in the 1940s, and effectively decreased morbidity and mortality Nevertheless, by the late 1940s, resistance due to the presence of penicillinase emerged (Eickhoff, 1972). Staphylococci may acquire resistance against common antimicrobial agents, including erythromycin (Wallmark, et al., 1961), ampicillin (Klein, et al., 1963), and tetracycline (Eickhoff, 1972). . In most cases, resistance to antibiotics is coded for by genes carried on plasmids, accounting for the rapid spread of resistant bacteria (Morris, et al., 1998). Immediately after methicillin was introduced (Jevons & Patricia, 1961), the emergence of MRSA was described, which have spread worldwide as a nosocomial pathogen. The Central Public Health Laboratory, (Cooke, et al., 2000) found that 61% isolates in

nosocomial *S. aureus* infections in the 96 hospitals studied were methicillin resistant. Penicillin, a ß-lactam antibiotic works by inhibiting bacterium cell wall synthesis by inactivating the penicillin-binding proteins (PBP). MRSA strains produce a distinct PBP, designated PBP2/, which has a low affinity to ß-lactam antibiotics, hence PBP2/ can still synthesis the cell wall in the presence of the antibiotic (Hiramatsu & Keiichi, 1995). This is the basis for ß-lactam resistance in MRSA strains. PBP2/ are products of the gene *mecA*. Foreign chromosomal DNA is found in methicillin resistant strains but not in methicillin susceptible strains. Vancomycin, a glycopeptide has been the most reliable antibiotic against MRSA infections; however, in 1996 the first MRSA to acquire vancomycin intermediate resistance was isolated in Japan (Hiramatsu, et al., 1997). Unfortunately, several vancomycin insensitive *S. aureus* (VISA) strains have been reported in the USA, France, Scotland, Korea, South Africa and Brazil. Upon exposure to vancomycin, certain MRSA strains frequently generate VISA strains, called hetero-VISA (Keiichi & Hiramatsu, 2001). VISA resistance appears to be associated with thickening of the cell wall peptidoglycan, and due to an increase in the target for the glycopeptide in the cell wall, therefore requiring more glycopeptide to inhibit the bacteria from growing (Hanaki, et al., 1998). All VISA strains isolated appear to have a common mechanism of resistance, which differs from that found in vancomycin resistant enterococci, in that enterococcal van genes are not present (Walsh & Christopher, 1993). However in 2002, the first vancomycin resistant *S. aureus* (VRSA) infection was documented in a patient in the United States (Center for Disease Control and Prevention (CDC), 2002). This strain was shown to carry *van* gene, suggesting that the resistance determinant might have been acquired through the genetic exchange of material between vancomycin resistant

enterococci and *S. aureus*. The spread of vancomycin resistance worldwide is now inevitable, and could potentially result in a return to pre-antibiotic era. Hence, the identification of novel targets on the bacteria seems to be a pre-requisite in the search for new antibiotics and prophylaxis, e.g. vaccines.

S. aureus has demonstrated a remarkable capacity to react rapidly to a new threat. *S. aureus* drug resistance is almost entirely regulated by the determinants obtained through horizontal DNA transfer to nearly all of the early antibiotic groups. The most alarming features of the susceptibility to methicillin and vancomycin are the lateral gene transfer in *S. aureus*. Endogenous resistance that is developed by the spontaneous mutation and antibiotic pressure selection cycle known to play a major role in the clinical community, and offers essential mechanisms of antibiotic resistance including fluoroquinolones, vancomycin, daptomycin, linezolid (Table 1). There is a broad variety of antimicrobial drugs used to manage infections with *S. aureus*, several of which remain usable.

Table 1: Mechanisms of antibiotic resistance for agents used in the treatment of *S. aureus* infections (Assis, et al., 2017).

Mechanisms of resistance to antibiotics employed for the treatment of *S. aureus* infections.

Antibiotic	Class	Mechanism of action	Mechanism of resistance
penicillin	β-lactam	Covalent link with PBPs	Acquisition of *mecA*, expression of low affinity PBP2a Hydrolysis by β-lactamases
ceftaroline	β-lactam (5th generation cephalosporin)	Covalent link with PBPs, including PBP2a	Introduction of mutations in PBP2a
vancomycin	glycopeptide	Binding to D-Ala-D-Ala stem peptides	Acquisition of *vanA*; downregulation of PBP4 expression
linezolid	oxazolidinone	Prevention of formation of the initiation complex on ribosome	Methylation of 23S rRNA ribosomal gene
Doxycycline, minocycline	tetracycline	Protein synthesis inhibition through binding to 30S subunit	Action of efflux pumps and ribosomal protection through RPPs
gentamicin	aminoglycoside	Protein synthesis inhibition through binding to 30S subunit	Methylation and mutation of 30S rRNA

S. aureus demonstrates two main antibiotic resistance pathways. The secretion of PC1 beta-lactamase at elevated rates requires one general process, while an antibiotic of beta-lactam is detected in the immediate cell environment. Vancomycin, ceftaroline and other glycopeptides are a mechanism of action involving the attachment by PBPs to the lipid II D-Ala-D-Ala C-terminal peptide, actual blocking of its recognition, and subsequent cross-linking (Walsh, et al., 2002). Two forms of resistant strains naturally emerged after widespread usage of vancomycin: (1) Vancomycin Intermediate *Staphylococcus aureus* (VISA) which displays an imperceptibly connected cell wall and (2) a vancomycin-resistant *S. aureus* (VRSA) which shows high degree of resistance to accumulation of D-Ala-D-Ala targets in the periphery of the cell. VISA strains typically arise due to prolonged vancomycin diagnosis due to bacterial pathogens, often contributing to inconsistent clinical outcomes. Doxycycline and minocycline are a broad-spectrum antibiotic community that attack the 30S ribosomal sub-unit to prevent protein synthesis. Tetracyclines are, as is the case with many other antimicrobials, natural product of the bacteria of active soils, but have had resistance problems, particularly in relation to efflux pumps and the action of RPPs, which weaken the interactive impact between antibiotics and ribosomal 30S (Table 1) (Poulakou, et al., 2014; Bassetti, et al., 2005).

MRSA strains are of particular concern as they are a leading global source of infections linked to health care and have also been shown to be the primary cause of community-based infections. MRSA contains the cassette chromosomal *mec* (SCC*mec*) as the staphylococcal tape, which represents a mobile genetic feature (Figure 6). This is the key determinant for broad-scale beta-lactam tolerance controlled by the *mecA* gene.

The production and incorporation of the methicillin-resistant staphylococcal lines into the chromosome of susceptible strains is attributed to SCC*mec*. *mecA*, located on SCC in *S. aureus* (21–67 kb fragment) (Noguchi, et al., 2005; Hiramatsu, et al., 2002), has also been called a genomic island or antibiotic resistance island (Katayama et al., 2000). Both SCC*mec* and non-*mec* SCC have been classified and characterized according to their putative cassette chromosome recombinase genes (*ccr*) and overall genetic composition (Hiramatsu, et al., 2001; Hiramatsu, et al., 2002; Ito, et al., 2003; Wisplinghoff, et al., 2003). SCC is a well-developed vehicle for genetic exchange of genes among staphylococcal species (Katayama, et al., 2003) and might be useful for cells living in various stressful environments. Integration of the element is sequence specific, i.e. at a unique site (bacterial chromosomal attachment site, attBSCC) located near the *S. aureus* origin of replication.

SCC*mec* carries specific genes (ccr), which encode recombinases of the invertase/resolvase family (Hiramatsu, et al., 2001; Ito, et al., 2004). Four different homologous pairs of ccrAB genes and one ccrC gene have been reported (Ito, et al., 2004; Hiramatsu, et al., 2002; Milheiricisco, et al., 2007). The Ccr catalytic motif at the N-terminal domain is characteristic of recombinases of the invertase/resolvase family (Abdel-Meguid, et al., 2001), and the catalytic serine residue of the recombination active site is conserved in all Ccr proteins (Hiramatsu, et al., 2001).Site specific recombinases of other bacterial genera are distantly related to the known *ccr* subfamilies, but their mode of action remains to be determined (Hiramatsu, et al., 2001; Ito, et al., 2004). SCC*mec* components are extremely complex and categorized according to their systemic structure

and genetic material. SCC*mec* elements contain a sequence of attachment site on bacterial chromosome (attBscc) which located near the replication origins at the end of 3' an open reading frame of uncertain function named termed orfX, which is well preserved in both MRSA and MSSA strains. The binding site includes a 15 bp core site sequence, the ISS integration site sequence that is required for ccr-mediated SCC*mec* chromosome-complex recombination, consisting of *mecA*-operon, *ccr* gene complex, cassette chromosome gene(s) and three regions bordering the *ccr* and *mec* complexes, known as joining regions (J), the following formulations are (orfX)J3-mec-J2-ccr-J1 composition.

mecA, its control genes and the related insertions form the *mec* gene set. The *mecA* gene complex is the prototype of a *mecA* complex which includes the entire regulatory genes mec R1 and mecI upstream of *mecA* and hypervariable area (HVR) as well as the IS431 upstream insert sequence of *mecA*. The B-Mec gene complex is composed of *mecA*, a *mecR1* truncated by addition of *mecA* upstream IS1272, HVR and mecA downstream IS431. The mec complex class C involves the *mecA* and mecR1 truncated by adding *mecA* and *mecA* upstream IS431 and *mecA* downstream of IS431. In class C1 mec, the *IS431* gene upstream *mecA* has the same orientation as IS431 downstream *mecA* (next to HVR), whereas in the class C2 mec complex mec is inverted the orientation of *IS431* upstream *mecA*. C1 and C2, although they were presumably formed separately, are seen as separate mec gene complexes. *mecA* and *mecR1*, however, does not contain a *mecR1* sequence (determined by PCR analysis), which runs downstream of *mecR1*.

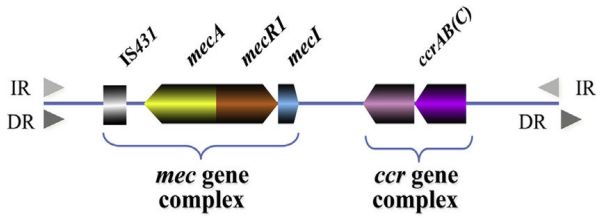

Figure 6: SCC*mec* consists of *mec*-gene complex and *ccr*-gene complex (Hiramatsu, et al., 2013).

There have been many variations identified in major *mec* gene complex groups, including the addition of *mecA* by *IS431* or *IS1182* in the *mecA* gene complex of class A or *mecA* upstream Tn4001 in the *mecA* complex of class B. The numerical string after the section shows these variations (Hiramatsu, et al., 2002; Hiramatsu, et al., 2001; Hanaki, et al., 1998) (Figure 7).

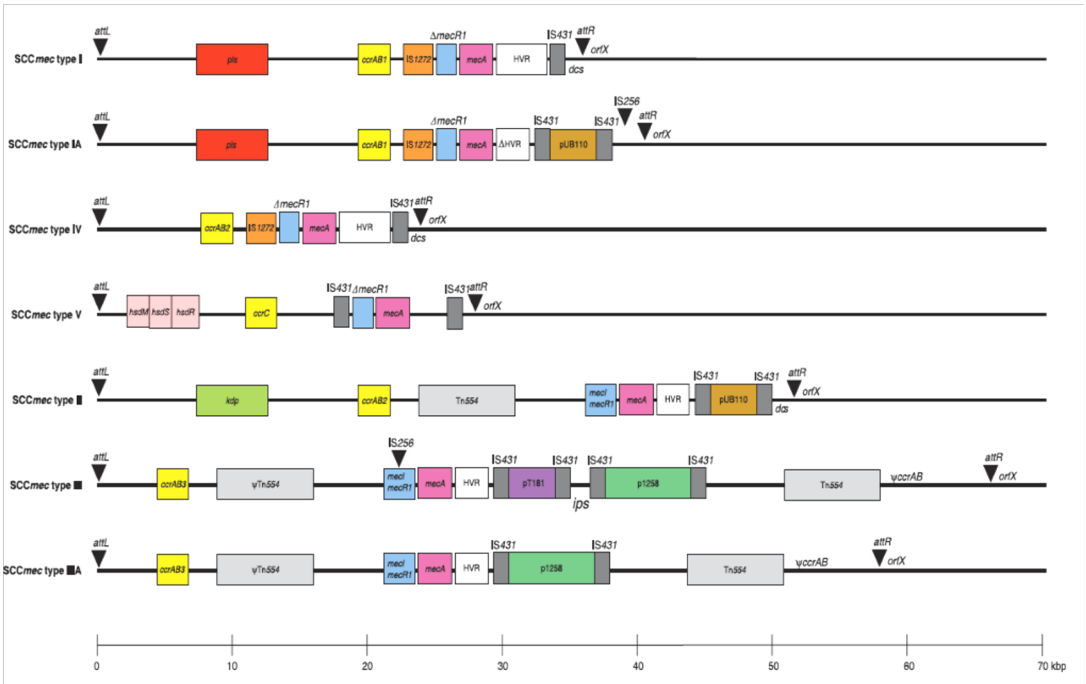

Figure 7: SCCmec elements type I-V observed in *staphylococci* (Johanna, et al., 2006).

SECTION TWO: MATERIALS AND METHODS

2.1 Bacterial Isolates

The samples included in this study were received at the Microbiology Laboratory at Near East University Hospital in Nicosia, Northern Cyprus. Fifty *S. aureus* isolates submitted to the laboratory between July 2012 to February 2020 and previously identified as MRSA by Phoenix 100 system (Becton Dickinson, BD Diagnostic Instrument Systems, USA) were randomly selected. Information on the isolation site for each sample such as skin, urine, blood, aspiration fluids, nasal swab, wound, abscess, and sputum, as well demographic data such as the age, gender and department of the patient were obtained from the patients' medical reports and recorded. Samples from all departments including Cardiology, Gastroenterology, Dialysis, Brain Surgery, General Surgery, Cardiovascular Surgery, Laboratory, Pediatrics, Plastic Surgery, Pulmonary Infections, Infectious Diseases, Orthopedics, Gynecology, Neurology, Urology, Dermatology and Intensive Care were included in this study.

2.2 Automatic Bacterial Identification System

Samples received in the laboratory which had growth upon bacterial culture were identified and antibiotic susceptibility testing was performed by Phoenix 100 (Becton Dickinson, BD Diagnostic Instrument Systems, USA) according to Antimicrobial

Susceptibility Testing (EUCAST) criteria. Bacterial suspensions from isolates grown on blood agar were prepared as 0.45-0.55 McFarland standard as recommended by the supplier. Phoenix NMIC/ID-400 and UNMIC/ID-401 panels were used. Isolates identified as *S. aureus* or MRSA species were stocked in cryopreservation solution containing %25 glycerol in -80 °C.

2.3 Bacterial Culture

All samples were processed by subculturing onto blood agar plates to obtain pure cultures. Blood agar base was prepared as directed by the supplier. Agar base was sterilized by autoclaving for 20 minutes at 121°, and were consequently placed in a 50°C water bath. When the agar base was cooled up a 50°C, 10% sterile blood (50 ml per 500 ml of agar base) was applied blended cautiously. 15 ml of media was aseptically poured into of sterile petri plates and were left to solidify at room temperature. Plates were stored in lined plastic bags at 2-8°C in order to avoid humidity loss. Glycerol stocks of isolates stored at -80°C, with designated stock number, were inoculated on freshly poured blood agar plates using sterile cotton applicators. The plates were incubated at 37°C for 24 h and examined for round, golden-yellow colonies.

2.4 Coagulate Test

Coagulase test was performed for each sample for confirmation of the newly retrieved bacterial samples. Coagulase test is performed to distinguish between *S. aureus*

and coagulase negative staphylococci. A few colonies of each bacterial culture were inoculated into sterile a glass tube containing 0.5 ml of fresh human plasma using a sterile cotton applicator and were gently mixed. Tubes were incubated at 37°C for 24 h and examined for clotting of the plasma inside the tube and recorded as coagulase positive. Tubes which showed slight clotting after 24 h incubation were incubated further for 4 h at room temperature and results were recorded. Inoculated tubes which did not any plasma clotting were recorded as coagulase negative and were not included in this study.

2.5 Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing for all isolates were performed in order to confirm methicillin resistant phenotype. Antibiotic susceptibility testing was performed using the disc diffusion assay method. A bacterial suspension per each sample by inoculating single colonies from pure cultures into sterile phosphate buffered saline (PBS) and the optical density was adjusted to 0.5 McFarland standard. The adjusted inoculum was transferred to freshly prepared Muller-Hinton agar (MHA) plates using sterile cotton applicators in order to form a lawn of bacterial growth. A cefoxitin disc (30 µg) was placed in the center of MHA plates and were incubated at 35°C for 24 h. Post incubation, zone of inhibition around the disc was measured using a millimetric ruler. Susceptibility was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Isolates were recorded as MRSA if the zone diameter was <22 mm and MSSA is zone diameter was ≥22 mm.

2.6 DNA Extraction

The isolates were grown on blood agar at 37 °C overnight, and genomic DNA was extracted from cultures using the boiling method. A few colonies were diluted in 500 µl sterile PBS in 1.5 eppendorf tubes, and were incubated at 100°C for 15 mins using a heat block, to lyse bacterial cells and free the DNA. After 15 mins of boiling, tubes were centrifuged at 13 000 g for 5 minutes to collect the lysed cells at the bottom of the tube. Supernatant containing genomic DNA was carefully transferred into a new sterile eppendorf tube and stored at -20°C until use.

2.7 Multiplex PCR Detection of *nuc* and *mecA*

A conventional gel-based multiplex PCR assay set up for simultaneous detection of *nuc* gene that encode *S. aureus* specific thermonuclease, and *mecA* gene. A conventional gel-based multiplex PCR assay set up for simultaneous detection of *nuc* and *mecA* genes. PCR reaction mix was prepared in a 25µl reaction volume, which included 12.5 µl of 2x Taq master mix (Thermo Scientific), 1 µl of each gene specific primer (mecA-F and mecA-R) and (nuc-F and nuc-R) at 10 µM concentration, 2 µl of template DNA and nuclease free water. Primer sets used for PCR amplification and the expected amplicon sizes are shown in Table 2.

Table 2: Primers that used for the amplification of *mecA/nuc* genes and amplicon sizes (Cunny & Witte, 2005)

Primer Name	Primer Sequence 5'→3'	Amplicon Size Base Pair (bp)
mecA-F	AAA ATC GAT GGT AAA GGT TGG C	533
mecA-R	AGT TCT GCA GTA CCG GAT TTG C	
nuc-F	GCG ATT GAT GGT GAT ACG GTT	270
nuc-R	AGC CAA GCC TTG ACG AAC TAA AGC	

The following PCR conditions were used: initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 30 sec composed of initial denaturation, and primer annealing at 55°C for 30 sec, and an extension of 72°C for 30 seconds and a final extension at 72°C for 10 mins. PCR reactions were performed using the Qiagen Rotor-Gene Q system. For each set of PCR, positive control for *mecA* and *nuc* genes, as well as the negative control (distilled water) was included. *S. aureus* SCC*mec* type IV strain (*nuc* +, *mecA* +, *pvl* -) from Aydın Adnan Menderes University, Recombinant DNA and Recombinant Protein Center (REDPROM) collection was used as amplification control. Agarose gel electrophoresis was performed to identify the presence or absence of both genes - a 530 bp band for *mecA* gene and a 270 bp band for *nuc* gene was recorded.

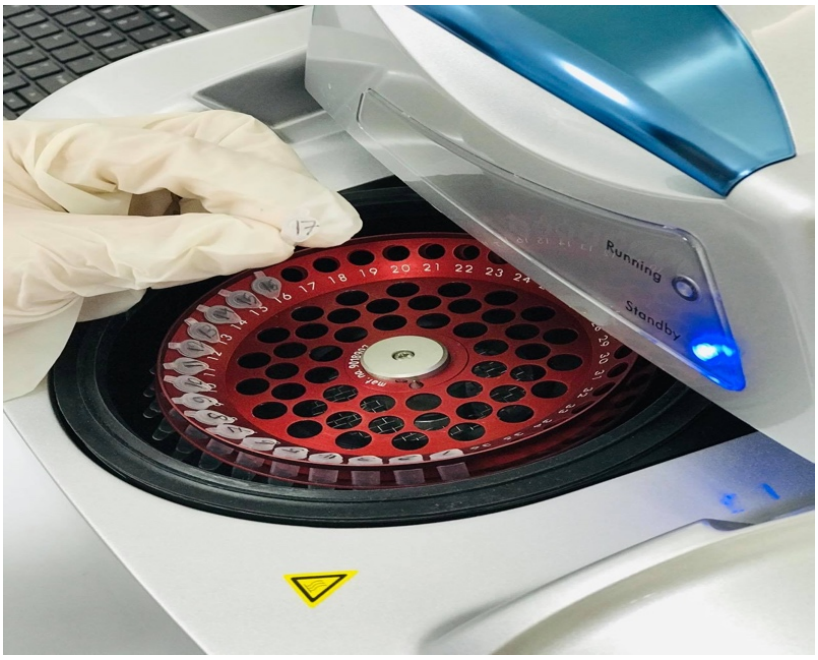

Figure 8: Polymerase chain reaction (PCR) is used in detecting *S. aureus* genes

2.8 PCR Detection of *pvl*

The prevalence of *pvl* gene was investigated for each sample using conventional PCR. PCR reaction mix was prepared in a 25µl reaction volume, which included 12.5 µl of 2X Taq master mix (Thermo Scientific), 2 µl of template DNA and 1 µl of each gene specific primer at 10 µM concentration. The primer sets used are shown in Table 3.

Table 3: The primers used for the amplification of pvl gene and amplicon size (Nawafl R., 2016)

Primer Name	Primer Sequence 5'→3'	Amplicon Size Base Pair (bp)
pvl-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433
pvl-R	GCATCAAGTGTATTGGATAGCAAAAGC	

All reactions contained a positive control (reference MRSA strain harboring *pvl* gene and negative control (nuclease free water). *S. aureus* SCC*mec* type II strain (*nuc* +, *mecA* +, *pvl* +) from Adnan Menderes University, Recombinant DNA and Recombinant Protein Center (REDPROM) collection was used as amplification control. Thermocycling parameters using the Qiagen Rotor-Gene Q system started with an initial denaturation at 94 °C for 10 min followed by a 35-cycle amplification program consisting of heating to 94 °C with a 30-s hold; annealing at 55 8C with a 30-s hold; and extension at 72 °C with a 30-s hold, and a final extension for 72°C 10 mins. The expected amplicon size was 433 bp for *pvl gene.*

2.9 Agarose Gel Electrophoresis

The PCR-amplified samples were analyzed by agarose gel electrophoresis by using a 2% (wt/vol) agarose gel in (200 ml) lx Tris/Borate/EDTA (TBE) buffer and with (20 ul) ethidium bromide incorporated for DNA staining. For the first well, 5 ul of 100 bp

DNA ladder (Hibrigen) was applied, and PCR samples (12.5 ul) were mixed with (2.5 ul) of 6x loading dye and loaded into each well. Gels were run in 1xTBE buffer at 120 V for 1 hour. PCR products were visualized using the DNA MiniBIS Pro Gel Imaging System.

Figure 9: Agarose gel electrophoresis carried out for analyzing amplified samples, and DNA MiniBIS Pro Gel Imaging System used for visualization of PCR products

2.10 Statistical Analysis

Data were analyzed using Microsoft Excel program version 16.37 and graphical representations were reported.

SECTION THREE: RESULTS

3.1 Patient characteristics

Fifty (50) clinical samples from patients admitted to Near East University Hospital were included in this study. Among the patient group, 72% inpatients (36/50) and 28% (14/50) outpatients (Figure 10). Of all the patients, 56% (28/50) were males and 44% (22/50) were females (Figure 11).

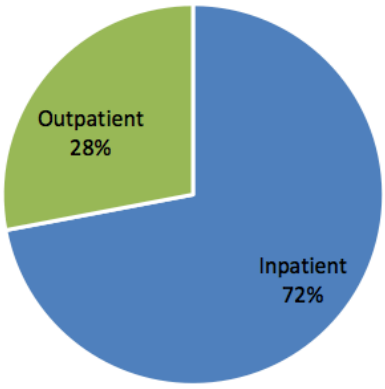

Figure 10: Type of admission for patients included in this study

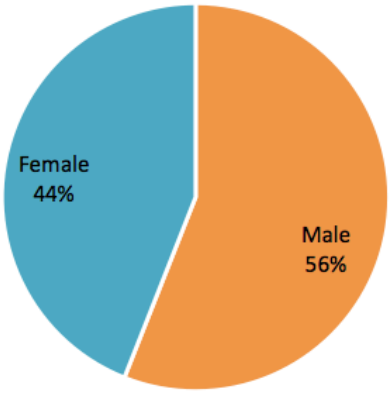

Figure 11: Gender of patients with MRSA infection included in this study

The age range of patients varied from 1 to 99 years of age, with highest number of cases observed in 60-69 and the lowest number of cases were detected in 10-19 age group. The distribution of MRSA cases according to age group is shown in Figure 12.

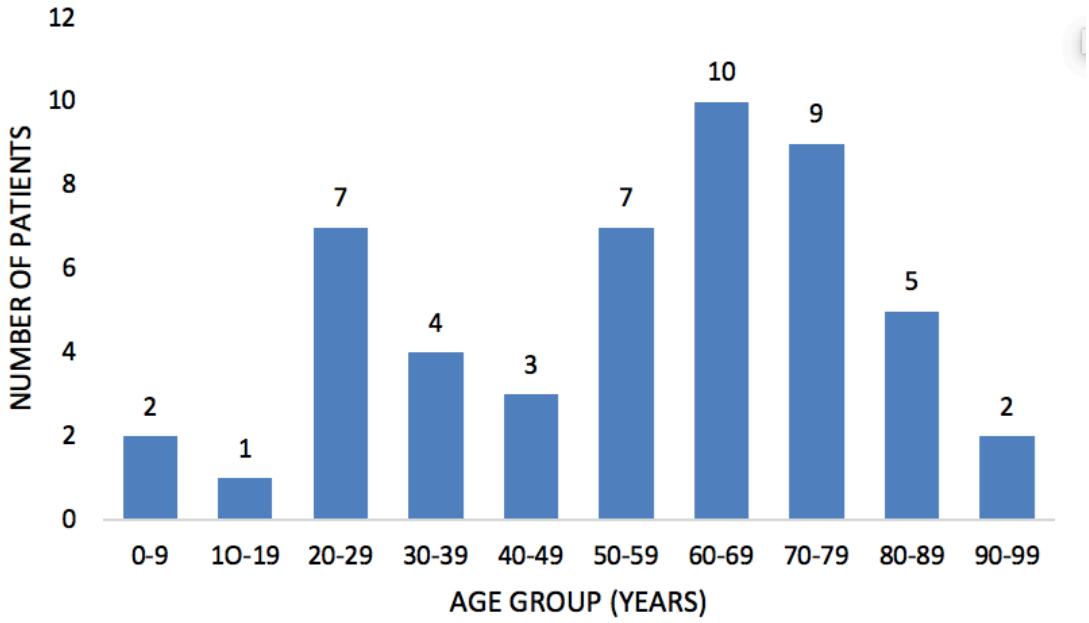

Figure 12: Distribution of patients with MRSA infection according to age group

Clinical samples from patients were obtained from a wide range of departments including Cardiology (22%, n=11), Pulmonary Infections (12%, n=6), Intensive Care (4%, n=2), Laboratory (8%, n=4), Neurology (6%, n=3), Orthopedics (8%, n=4), Gastroenterology (6%, n=3), General Surgery (6%, n=3), Brain Surgery (4%, n=2), Cardiovascular Surgery (4%, n=2), Dermatology (4%, n=2), Dialysis (2%, n=1), Plastic Surgery (2%, n=1), Pediatrics (2%, n=1), Infectious Diseases (2%, n=1), Gynecology (2%, n=1) and Urology (4%, n=2) were included in this study. The highest number of

MRSA cases were observed in Cardiology and Pulmonary Infections departments followed by the Intensive Care Unit and Laboratory. Distribution of cases according to hospital departments is shown in Figure 13.

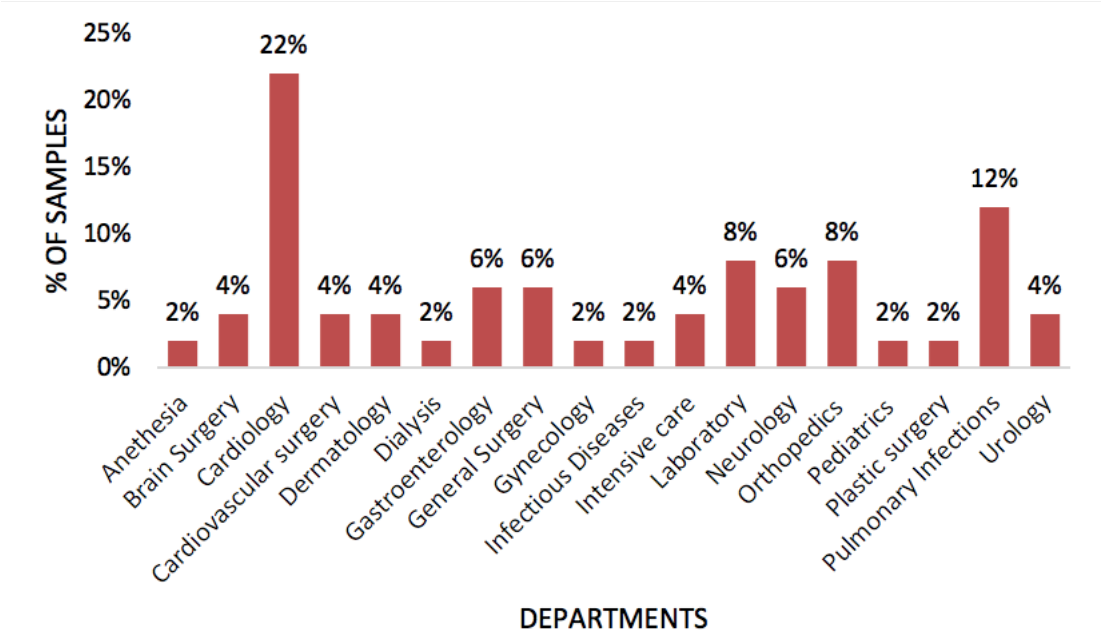

Figure 13: Distribution of MRSA cases according to hospital departments

3.2 Isolation site

The clinical samples were from collected from diverse body sites of patients. Analysis of sample isolation sites indicated that 22% (n=11) of the samples were taken from abscess/wound, 18% (n=9) deep tracheal aspirate (DTA), 18% (n=9) nasal swab, 16% (n=8) of the samples were from blood, 10% (n=5) urine, 4% (n=2) sputum, 4% (n=2) catheter tips, 4% (n=2) body fluids, 4% (n=2) bronchioalveolar lavage and 2% (n=1) were

from urethral origin. Data suggested that the highest number of MRSA cases were observed in samples isolated from abscess/wound, followed by DTA and blood. Analysis of different isolation sites for all samples are shown in Figure 14.

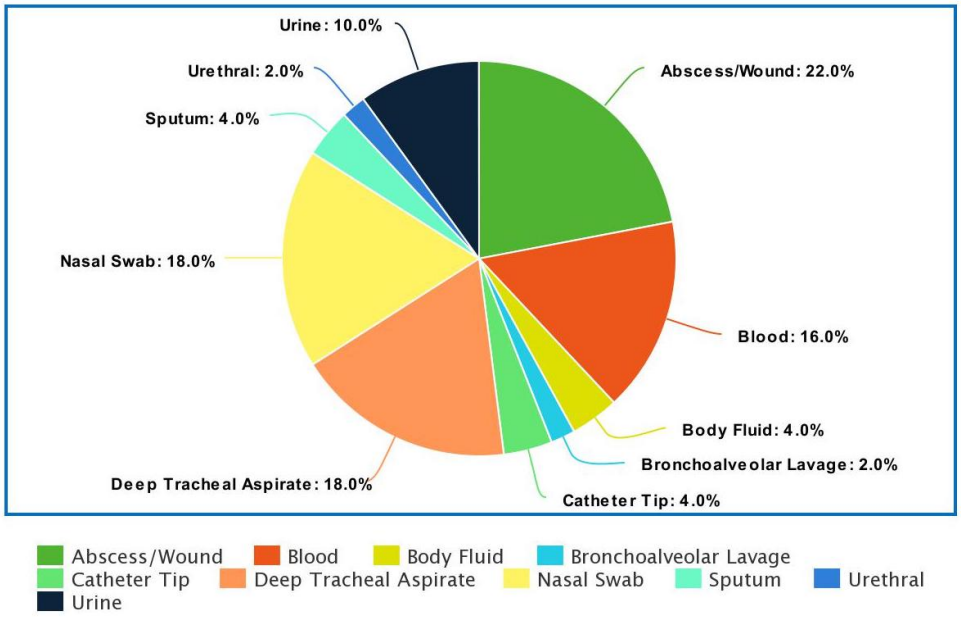

Figure 14: Analysis of different body sites as a source of MRSA infection

3.3 Coagulase test

All 50 *S. aureus* samples included in this study were initially cultured on fresh blood agar plates and examined for golden-yellow *S. aureus* colonies (Figure 15) and consequently tested with tube coagulase assay in order to rule out coagulase negative staphylococci isolates. All bacterial isolates tested were found to be coagulase positive (Table 4).

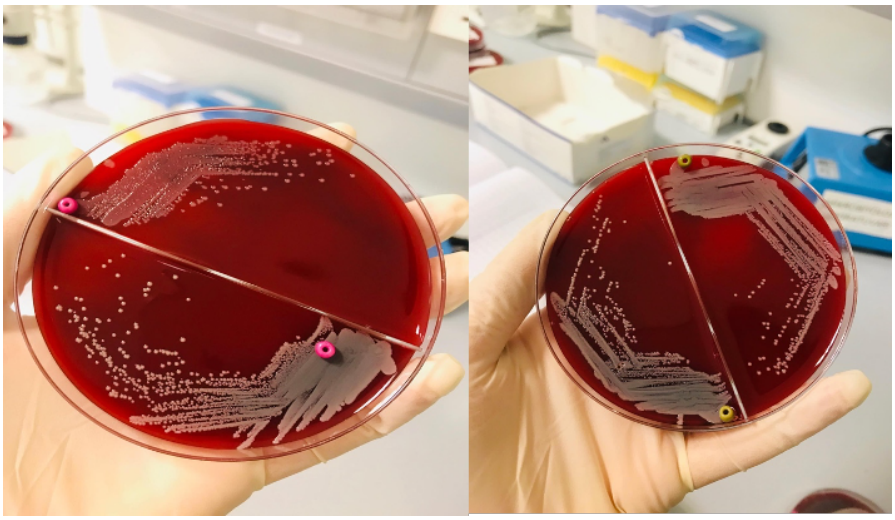

Figure 15: Golden-yellow *S. aureus* colonies on blood agar plates after 24 h incubation at 37 ˚C

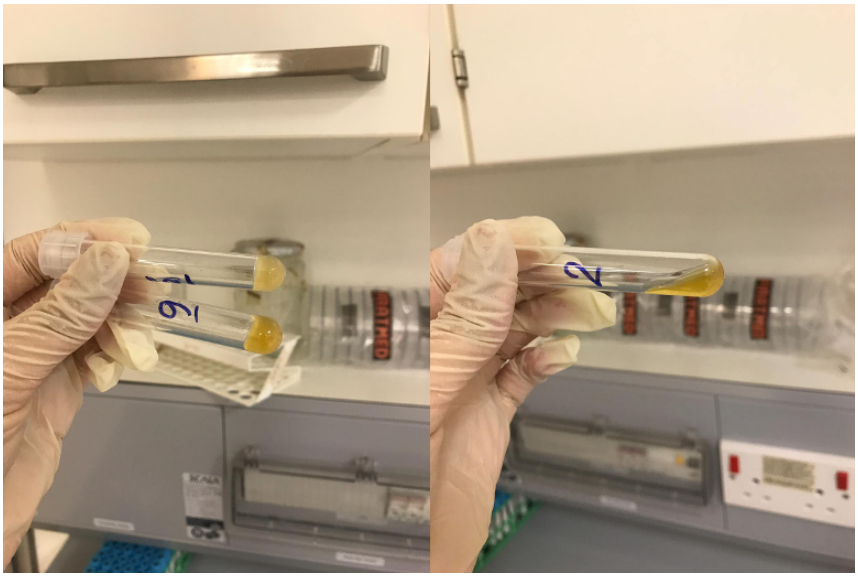

Figure 16: Coagulase test of *Staphylococcus spp*. The left tubes show positive results (the plasma inside the tubes has coagulated) and the right tube shows a negative result.

Table 4: Data table for all MRSA isolates used in this study

Isolate No.	Gender	Age	Isolation Site	Inpatient/Outpatient	Organism	Department	Coagulase	Cefoxitin (mm)	nuc PCR	mecA PCR	PVL PCR	MRSA/MSSA
Patient 1	female	79	Nasal swab	Inpatient	*S. aureus*	Cardiology	Positive	17	positive	negative	-	MRSA
Patient 2	male	73	Nasal swab	Inpatient	*S. aureus*	Cardiology	Positive	18	positive	negative	-	MRSA
Patient 3	female	74	Nasal swab	Inpatient	*S. aureus*	Cardiology	Positive	17	positive	negative	-	MRSA
Patient 4	female	69	Abscess/wound	Inpatient	*S. aureus*	Gastroenterology	Positive	15	positive	positive	-	MRSA
Patient 5	male	70	Abscess/wound	Inpatient	*S. aureus*	Cardiology	Positive	16	positive	negative	-	MRSA
Patient 6	female	76	Abscess/wound	Inpatient	*S. aureus*	Dialysis	Positive	16	positive	positive	-	MRSA
Patient 7	male	19	Deep tracheal aspirate	Inpatient	*S. aureus*	Brain Surgery	Positive	16	positive	positive	-	MRSA
Patient 8	male	41	Blood	Inpatient	*S. aureus*	Pulmonary Infections	Positive	18	positive	negative	-	MRSA
Patient 9	female	80	Sputum	Inpatient	*S. aureus*	Cardiology	Positive	14	positive	positive	-	MRSA
Patient 10	male	63	Body fluid	Outpatient	*S. aureus*	Gastroenterology	Positive	15	positive	positive	-	MRSA
Patient 11	female	63	Nasal swab	Outpatient	*S. aureus*	Laboratory	Positive	14	positive	positive	-	MRSA
Patient 12	male	25	Nasal swab	Outpatient	*S. aureus*	Laboratory	Positive	20	positive	positive	-	MRSA
Patient 13	male	47	Blood	Inpatient	*S. aureus*	General Surgery	Positive	14	positive	positive	-	MRSA
Patient 14	male	63	Abscess/wound	Outpatient	*S. aureus*	Orthopedics	Positive	18	positive	negative	-	MRSA
Patient 15	male	67	Blood	Inpatient	*S. aureus*	Infectious Diseases	Positive	17	positive	negative	-	MRSA
Patient 16	female	58	Deep tracheal aspirate	Inpatient	*S. aureus*	Laboratory	Positive	14	positive	positive	-	MRSA
Patient 17	male	60	Blood	Inpatient	*S. aureus*	Gastroenterology	Positive	13	positive	positive	-	MRSA
Patient 18	male	27	Catheter tip	Inpatient	*S. aureus*	Neurology	Positive	15	positive	negative	-	MRSA
Patient 19	female	80	Sputum	Inpatient	*S. aureus*	Pulmonary Infections	Positive	13	positive	positive	-	MRSA
Patient 20	female	80	Deep tracheal aspirate	Inpatient	*S. aureus*	Pulmonary Infections	Positive	14	positive	positive	-	MRSA
Patient 21	male	53	Urine	Inpatient	*S. aureus*	Cardiology	Positive	13	positive	positive	-	MRSA
Patient 22	male	20	Abscess/wound	Outpatient	*S. aureus*	Orthopedics	Positive	15	positive	positive	-	MRSA
Patient 23	female	25	Urine	Outpatient	*S. aureus*	Gynecology	Positive	19	positive	positive	PVL	MRSA
Patient 24	female	99	Deep tracheal aspirate	Inpatient	*S. aureus*	Pulmonary Infections	Positive	16	positive	positive	PVL	MRSA
Patient 25	male	65	Blood	Inpatient	*S. aureus*	Neurology	Positive	12	positive	negative	-	MRSA
Patient 26	male	65	Blood	Inpatient	*S. aureus*	Neurology	Positive	15	positive	negative	-	MRSA
Patient 27	male	66	Catheter tip	Inpatient	*S. aureus*	Cardiology	Positive	10	positive	positive	PVL	MRSA
Patient 28	male	55	Nasal swab	Inpatient	*S. aureus*	Cardiology	Positive	18	positive	negative	-	MRSA
Patient 29	female	58	Bronchoalveolar lavage	Inpatient	*S. aureus*	Pulmonary Infections	Positive	11	positive	positive	PVL	MRSA
Patient 30	male	75	Blood	Inpatient	*S. aureus*	Cardiology	Positive	16	positive	positive	-	MRSA
Patient 31	female	77	Nasal swab	Inpatient	*S. aureus*	Cardiovascular surgery	Positive	15	positive	positive	-	MRSA
Patient 32	male	34	Nasal swab	Inpatient	*S. aureus*	Cardiology	Positive	16	positive	positive	PVL	MRSA
Patient 33	male	87	Deep tracheal aspirate	Inpatient	*S. aureus*	Anethesia	Positive	13	positive	positive	-	MRSA
Patient 34	female	1	Urine	Outpatient	*S. aureus*	Pediatrics	Positive	15	positive	positive	PVL	MRSA
Patient 35	female	63	Nasal swab	Inpatient	*S. aureus*	Cardiovascular surgery	Positive	15	positive	positive	PVL	MRSA
Patient 36	male	41	Deep tracheal aspirate	Inpatient	*S. aureus*	Pulmonary Infections	Positive	16	positive	negative	PVL	MRSA
Patient 37	female	85	Deep tracheal aspirate	Inpatient	*S. aureus*	Brain surgery	Positive	16	positive	positive	-	MRSA
Patient 38	female	59	Abscess/wound	Inpatient	*S. aureus*	Orthopedics	Positive	20	positive	positive	-	MRSA
Patient 39	male	76	Blood	Inpatient	*S. aureus*	Cardiology	Positive	17	positive	positive	-	MRSA
Patient 40	male	55	Abscess/wound	Outpatient	*S. aureus*	Dermatology	Positive	19	positive	positive	PVL	MRSA
Patient 41	female	36	Urine	Outpatient	*S. aureus*	Laboratory	Positive	15	positive	positive	-	MRSA
Patient 42	female	20	abscess/wound	Outpatient	*S. aureus*	Dermatology	Positive	13	positive	positive	PVL	MRSA
Patient 43	male	22	Urethral	Outpatient	*S. aureus*	Urology	Positive	13	positive	positive	-	MRSA
Patient 44	female	28	Abscess/wound	Inpatient	*S. aureus*	General surgery	Positive	18	positive	positive	PVL	MRSA
Patient 45	female	1	Abscess/wound	Outpatient	*S. aureus*	Plastic surgery	Positive	18	positive	negative	-	MRSA
Patient 46	female	96	Deep tracheal aspirate	Inpatient	*S. aureus*	Intensive care	Positive	16	positive	positive	PVL	MRSA
Patient 47	male	79	Deep tracheal aspirate	Inpatient	*S. aureus*	Intensive care	Positive	16	positive	positive	PVL	MRSA
Patient 48	male	37	Abscess/wound	Outpatient	*S. aureus*	General surgery	Positive	30	positive	negative	PVL	MSSA
Patient 49	male	55	Urine	Inpatient	S. aureus	Urology	Positive	28	positive	negative	PVL	MSSA
Patient 50	male	31	Body fluid	Outpatient	S. aureus	Orthopedics	Positive	27	positive	negative	-	MSSA

3.4.1. Cefoxitin Disc Diffusion Assay

All 50 *S. aureus* isolates confirmed with coagulase assay were subjected to cefoxitin disc diffusion assay in order to confirm the methicillin resistant phenotype of the isolates detected by the Phoenix 100 system (Becton Dickinson, BD Diagnostic Instrument Systems, USA). Susceptibility was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Isolates were recorded as MRSA if the zone diameter was < 22 mm and MSSA if zone diameter was ≥ 22 mm. Results of the cefoxitin disc diffusion assay are shown in Table 4. Forty-seven (n=47) of the isolates were found to be cefoxitin resistant (MRSA), while three isolates (48, 49, 50) were detected to be susceptible to cefoxitin (MSSA) with zone diameters of 30 mm, 28 mm and 27 mm respectively.

3.4.2. Amplification of *nuc* and *mecA* Genes

A multiplex PCR was used for the simultaneous detection of *nuc* and *mecA* genes in gDNA from all isolates. Out of 50 samples, 100 % (n=50) were *nuc* positive. All of the isolates that were positive for the *nuc* gene by PCR, with a 270 bp band detected by agarose gel electrophoresis, were confirmed as *S. aureus*. Among the *nuc* positive isolates, 68% (n=34) were *mecA* positive by PCR with a 533 bp on agarose gel electrophoresis (Table 4). The remaining 13 isolates (26%) (excluding three isolates no. 48, 49, 50) determined to be MSSA by the cefoxitin susceptibility assay), required further testing by *mecC* PCR to identify the mechanism of methicillin resistance in these isolates.

A representative gel image of the multiplex PCR for *nuc* and *mecA* genes is shown in Figure 17.

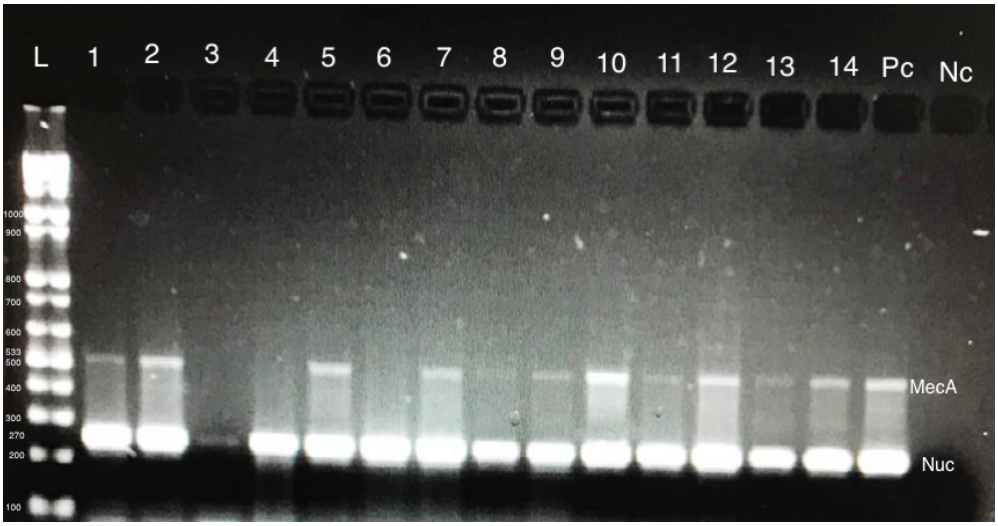

Figure 17: Detection of *nuc* and *mecA* amplification in *S. aureus* isolates by multiplex PCR. Agarose gel shows positive results for *nuc* gene amplification as a 270 bp amplicon, and positive results for *mecA* gene amplification as a 533 bp amplicon

3.5 Amplification of *pvl* Gene

Of the 50 isolates tested, 30% (n=15) were *pvl* positive by PCR as demonstrated by the amplification of a 433 bp band. As three of the isolates (isolates no. 48, 49, 50) were determined to be MSSA by the cefoxitin susceptibility assay, they were excluded from the PVL prevalence calculations and the total number of MRSA isolates were taken as 47. Therefore, the prevalence of PVL in MRSA isolates at Near East University

Hospital tested was determined to be 27.7% (13/47). Amplification of *pvl* in all tested isolates are shown in Figures 18-21.

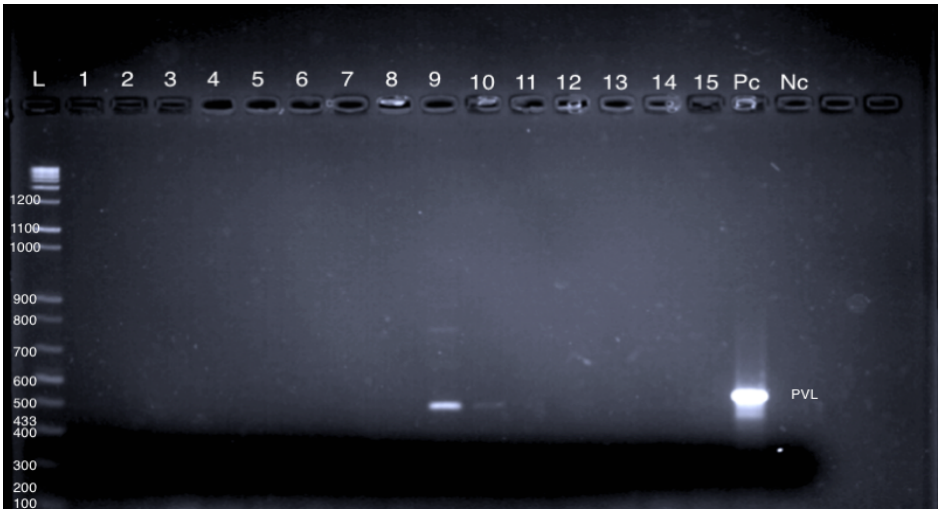

Figure 18: Detection of the *pvl* gene in MRSA isolates #1-15 demonstrated by the amplification of 433 bp band. Two samples were positive excluding the positive control (PC), negative control (NC) confirmed lack of contamination. 100bp DNA ladder (L).

Table 5: *pvl* amplification of isolates #1-15

#	Isolate No.	*pvl* Amplification	Remarks
1	Patient 1	Negative	MRSA
2	Patient 2	Negative	MRSA
3	Patient 3	Negative	MRSA
4	Patient 4	Negative	MRSA
5	Patient 5	Negative	MRSA
6	Patient 6	Negative	MRSA
7	Patient 7	Negative	MRSA
8	Patient 8	Negative	MRSA
9	Patient 9	Positive	MRSA

10	Patient 10	Positive	MRSA
11	Patient 11	Negative	MRSA
12	Patient 12	Negative	MRSA
13	Patient 13	Negative	MRSA
14	Patient 14	Negative	MRSA
15	Patient 15	Negative	MRSA

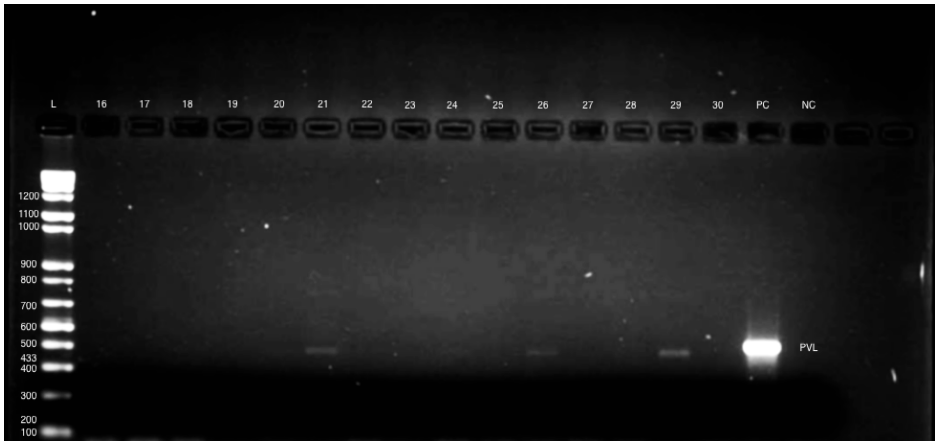

Figure 19: Detection of the *pvl* gene in MRSA isolates #16-30 demonstrated by the amplification of 433 bp band. Three samples were positive, two samples were weak positive. Positive control (PC), negative control (NC), 100bp DNA ladder (L).

Table 6: *pvl* amplification of isolates #16-30

#	Isolate no.	*pvl* amplification	Remarks
16	Patient 16	Negative	MRSA
17	Patient 17	Negative	MRSA
18	Patient 18	Negative	MRSA
19	Patient 19	Negative	MRSA

20	Patient 20	Weak Positive	MRSA
21	Patient 21	Positive	MRSA
22	Patient 22	Negative	MRSA
23	Patient 23	Negative	MRSA
24	Patient 24	Weak Positive	MRSA
25	Patient 25	Negative	MRSA
26	Patient 26	Positive	MRSA
27	Patient 27	Negative	MRSA
28	Patient 28	Negative	MRSA
29	Patient 29	Positive	MRSA
30	Patient 30	Negative	MRSA

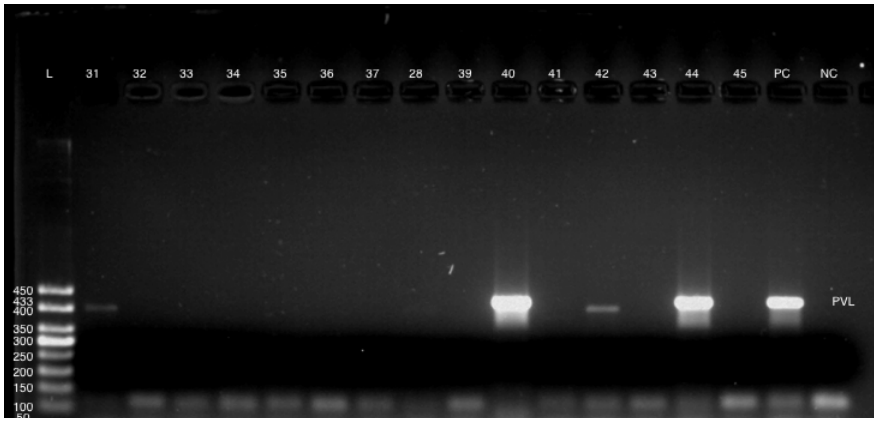

Figure 20: Detection of *the pvl* gene in MRSA isolates #31-45 demonstrated by the amplification of 433 bp band. Four samples were positive. Positive control (PC), negative control (NC), 50 bp DNA ladder (L)

Table 7: *pvl* amplification of isolates #31-45

#	Isolate no.	*pvl* amplification	Remarks
31	Patient 31	Positive	MRSA
32	Patient 32	Negative	MRSA
33	Patient 33	Negative	MRSA
34	Patient 34	Negative	MRSA
35	Patient 35	Negative	MRSA
36	Patient 36	Negative	MRSA
37	Patient 37	Negative	MRSA
38	Patient 38	Negative	MRSA
39	Patient 39	Negative	MRSA
40	Patient 40	Positive	MRSA
41	Patient 41	Negative	MRSA
42	Patient 42	Positive	MRSA
43	Patient 43	Negative	MRSA
44	Patient 44	Positive	MRSA
45	Patient 45	Negative	MRSA

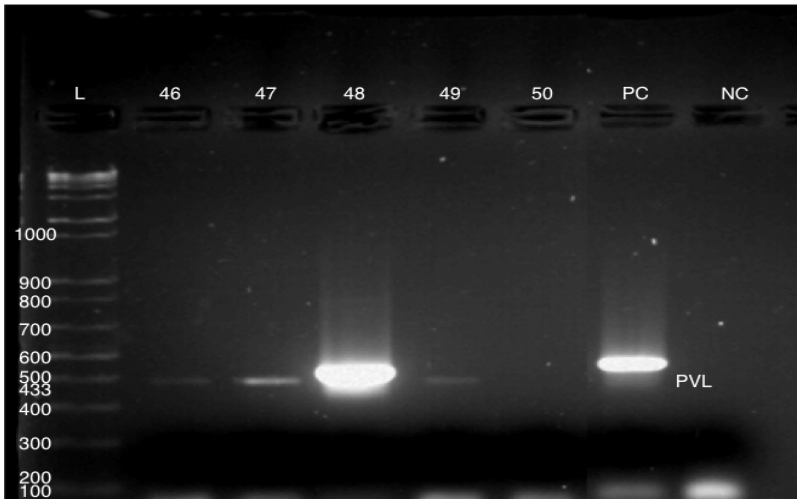

Figure 21: Detection of the *pvl* gene in MRSA isolates #46-50 demonstrated by the amplification of 433 bp band. Four samples were positive. 100 bp DNA ladder (L)

Table 8: *pvl* amplification of isolates #46-50

#	Isolate no.	*pvl* amplification	Remarks
46	Patient 46	Positive	MRSA
47	Patient 47	Positive	MRSA
48	Patient 48	Positive	MSSA
49	Patient 49	Positive	MSSA
50	Patient 50	Negative	MSSA

SECTION FOUR: DISCUSSION

Virulence potential of all pathogenic microorganisms is a major indicator of the clinical characteristics and severity of disease. Virulence factors enable pathogens to colonize and invade host tissues, adapt to host milieu, subvert host functions and overcome host defense mechanisms. Among the plethora of virulence factors of *S. aureus*, PVL plays an important role in in the pathogenesis of tissue injury and inflammation. The emerging prevalence of PVL-positive MRSA is a major health concern worldwide. Before 2000, PVL genes were rarely observed in HA-MRSA isolates, and were commonly detected in and considered as unambiguous markers of CA-MRSA. More recently, HA-MRSA isolates were also confirmed to carry PVL genes. PVL is known to be a main virulence factor in MRSA strains, particularly in CA-MRSA infections, such as pneumonia necrotizing, skin or soft tissue infection. Such disease agents are recognized for their significant tissue necrosis and leukopenia, which are related to the capacity of PVL to destroy neutrophils, the main defense cells against invading bacteria (Varshney, et al., 2010). The increasing prevalence of PVL-encoding HA-MRSA is a serious concern which is considered to worsen the outcome MRSA infections in patients.

In the present study, fifty MRSA clinical isolates from patients admitted to Near East University Hospital in Northern Cyprus were investigated for the presence of PVL gene using molecular methods. The prevalence of PVL-positive MRSA was determined to be 27.7%. Out of 72% of inpatient MRSA isolates 69.2% were PVL-positive, however

among 28% of outpatient MRSA isolates 30.8% were PVL-positive. Among 50 samples from patients were obtained from a wide range of departments including Cardiology (22%, n=11), Pulmonary Infections (12%, n=6), Intensive Care (4%, n=2), Laboratory (8%, n=4), Neurology (6%, n=3), Orthopedics (8%, n=4), Gastroenterology (6%, n=3), General Surgery (6%, n=3), Brain Surgery (4%, n=2), Cardiovascular Surgery (4%, n=2), Dermatology (4%, n=2), Dialysis (2%, n=1), Plastic Surgery (2%, n=1), Pediatrics (2%, n=1), Infectious Diseases (2%, n=1), Gynecology (2%, n=1) and Urology (4%, n=2) were included in this study. The highest number of MRSA cases were observed in Cardiology and Pulmonary Infections departments followed by the Intensive Care Unit. Among the patient groups, 72% (36/50) were inpatients and 28% (14/50) were outpatients. The age range of patients varied from 1 to 99 years of age, with highest number of cases observed in 60-69 and the lowest number of cases were detected in 10-19 age group. Of all the patients, 56% (28/50) were males and 44% (22/50) were females. Samples of *S. aureus* strains were isolated from various sites of patients. As analysis showed, isolation sites indicated that 22% (n=11) of the samples were taken from abscess/wound, 16% (n=8) of the samples were from blood, 18% (n=9) deep tracheal aspirate (DTA), 18% (n=9) nasal swab, 10% (n=5) urine, 4% (n=2) sputum, 4% (n=2) catheter tips, 4% (n=2) body fluids, 4% (n=2) bronchioalveolar lavage and 2% (n=1) were from urethral origin. Data suggested that the highest number of MRSA cases were observed in samples isolated from abscess/wound, followed by DTA and blood from inpatients and outpatients of Near East University Hospital. Among the PVL-positive MRSA isolates 76.9 % (10/13) were inpatients. PVL positivity was more common in MRSA isolated from DTA (38.5%, 5/13) and abscess/wound (23.1%, 3/13). The PVL-positive isolates were from 9 ward areas, but

were mainly from the Pulmonary Infections and Dermatology departments, as well as the Intensive Care Unit.

After 2003, the occurrence of CA-MRSA in Europe has attracted attention; retrospective analysis nevertheless suggest that events existed occasionally before. In terms of virulence gene expression, other countries should also be considered. In Germany, four out of eighteen patients with MRSA have demonstrated PVL-positive occurrence in dermatologic outpatients with inflammatory skin disorders, leg ulcers and skin infections in the ambulatory department of Heidelberg University (Jappe, et al., 2008). In general, the German National Reference Center (NRC) identified PVL-positivity to be 207 (1.74%) of 12 350 in MRSA isolates of different origin from 2003 to 2006 (Witte, et al., 2007). In 2003 and 2004, analysis performed in France of 238 patients admitted into emergency indicated that 93 of them (39%) were positive for MRSA; 84 were reported to have at least one hospital stay over the 12 months preceding the observation. Seven of the 93 patients (2.9%) had PVL-positive MRSA isolates (Viallon, et al., 2007). In Turkey, PVL-positivity in MRSA isolates in hospitals was reported to range between 6.9-12.7% (Akouglu, et al., 2010; Oksuz, et al., 2013). In a 2010 study in England and Wales, program of enhanced surveillance of PVL-MRSA indicated an increase trend in clinical specimens (Ellington, et al., 2010), with reports of PVL-positive MRSA outbreak in regional neonatal unit in the UK in 2012 (Ali, et al., 2012). Alternatively, in Spain, presence of PVL among MRSA isolates has also reported (Ana, et al., 2009; Vindel, et al., 2014). In a study conducted in Ireland, the prevalence of *pvl*-positive MRSA isolates submitted to National MRSA Reference Laboratory was reported to be 8.8% in 2011 (Shore, et al., 2014). On the other hand, the prevalence of PVL gene

among MRSA strains is reported to be 5.3% in 2011 in Malaysia (San, et al., 2017), whereas PVL-positivity was as low as 0.5% among MRSA isolates collected in a tertiary care hospital in Japan (Aung, et al., 2019). D'Souza et al has characterized MRSA isolates from a collection representing a mixed hospital- and community-associated patient population in India, and has reported *pvl* positivity to be 56.7%. In an attempt to investigate MRSA isolates in developing countries, Shrestha et al has screened MRSA isolates at a tertiary hospital in Nepal, and indicated PVL gene positivity rate in nosocomial MRSA isolates to be 26.1% (Shrestha, et al., 2014). Alternatively, PVL-MRSA has been also linked nosocomial infections in which hospital transmission of CA-MRSA had occurred in the US (Saiman, et al., 2003), highlighting the important source of CA-MRSA as a public health threat in hospitals. In China, 35.6 % of the nosocomial *S. aureus* gene carriage levels was observed for PVL: 26.1 % for MRSA and 51.9 % for MSSA. Such data represent some of the highest reported levels of PVL-positive *S. aureus* and should be viewed as a significant matter of concern. Such results, along with elevated incidence levels of PVL-positive *S. aureus*, should trigger infection prevention strategies enforced immediately (Shrestha, et al., 2014).

MRSA identification can be performed via broth-based and agar-based methods. Disc diffusion or the Kirby-Bauer method is a common technique in microbiology and still widely performed, because it is flexible and allows visibility of inhibition zones. The disc diffusion approach is potentially the most widely used system for evaluating antimicrobial resistance worldwide, owing to its convenience, reliability and low cost. In this study, three isolates identified as MRSA with the Phoenix 100 system (Becton

Dickinson, BD Diagnostic Instrument Systems, USA) has been detected to be susceptible to cefoxitin by disc diffusion assay depending on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines with zone diameters of 30 mm, 28 mm and 27 mm respectively. The observed discordance has been previously reported in other studies (Mencacci, et al., 2009).

Detection of the *mecA* gene or PBP2a, on the other hand, is considered the gold standard to identifying *mecA*-mediated methicillin resistance in staphylococci. Molecular techniques, based primarily on PCR, have become an important source for diagnosing infectious diseases. Although PCR is highly sensitive and specific, there are factors that may cause inaccuracy. More sensitive means more specific to its experimental condition. Many parameters affect PCR sensitivity, such as primers concentration, enzyme, temperature and cycles. All these parameters must be reviewed step by step. A major factor impacting sensitivity is the amount of copies of the DNA target in the microorganism's genome. The nucleic acid extraction process and genetic variation of the DNA target involve certain variables influencing PCR response. Differing efficacy of the extraction methods can have a substantial influence on the sensitivity (Rajal, et al., 2007).

Although targeting a significant virulence potential of an infectious pathogen, this study may have limitations such as the sample size. This study can be improved by increasing the number of clinical isolates. The quantity of samples is important consideration for research, as this provides a more accurate mean value and identify the outlier that can distort the data in a smaller amount and provide a smaller amount of error.

Increasing the volume of samples will help increasing the number of findings we achieve in the studies, meanwhile providing a better environment for utilizing more suitable technics in the statistics. A comparison of PVL expression in CA-MRSA versus HA-MRSA would also be helpful in understanding the variation in terms of prevalence of virulence genes among isolates of different origin.

SECTION FIVE: CONCLUSION

In the current study, the prevalence of PVL-positive MRSA at a tertiary hospital in Northern Cyprus was determined to be 27.7%. The prevalence of PVL among the MRSA isolates was relatively high particularly among deep aspirate tracheal and abscess/wound samples suggesting an important function for PVL played by pyogenic infections in the population, mainly skin and soft tissue infections (SSTIs). The combination of the PVL gene with the *mecA* gene as a resistance factor makes *S. aureus* a well-adapted pathogen. Problems have emerged in the treatment of MRSA infections over the years according the improper use of antibiotics, which limits therapeutic choice. In multidrug-resistant bacteria such as MRSA, the existence of PVL may be correlated with virulence and may escalate clinician challenges. Continued surveillance and characterization of MRSA isolates in hospitals in the country is imperative for the prevention of spread of virulent nosocomial infections and the implementation of enhanced infection control strategies. Further in-depth molecular typing of clinical MRSA isolates should also be pursued to identify MRSA SCC*mec* types circulating both in the community and hospital setting in Northern Cyprus.

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