OCCURENCE OF γ-HEMOLYSIN AND PANTON-
VALENTINE LEUKOCIDIN GENES AND
ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF
STAPHYLOCOCCUS AUREUS ISOLATED FROM
CLINICAL SAMPLES IN LEBANON

by

Pamela F. Abou Khalil

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Thesis Advisor: Dr. Sima Tokajian

Member   :   Dr. Fuad Hashwa

Member   :   Dr. Roy Khalaf

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Pamela Abou Khalil

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ABSTRACT

OCCURRENCE OF γ-HEMOLYSIN AND PANTON-VALENTINE LEUKOCIDIN GENES AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL SAMPLES IN LEBANON

by Pamela F. Abou Khalil

Staphylococcus aureus is a major causative agent of infections from mild superficial skin infection to life-threatening bacteraemia and infective endocarditis. The pathogenicity of *S. aureus* infections is correlated with extracellular proteins including hemolysins and Panton-Valentine Leukocidin (PVL). PVL and γ-hemolysin are members of a toxin family known as synergohymenotropic toxins, since both of them act on the cell membrane by the synergy of two proteins that form a pore. PVL is associated with disease states that range from superficial skin infections to severe septicemia. A single duplex PCR assay was used in this study, to investigate the prevalence of PVL and γ-hemolysin genes among 145 *S. aureus* clinical isolates recovered at major tertiary care medical centers in Lebanon. The majority (91%) of the isolates harbored the γ-hemolysin gene and 45% of the isolates harbored the PVL gene. PVL gene predominated in males below the age of 55 years old. Wounds were the predominant site of staphylococcal infections, with 52% of those strains harboring the PVL gene. PVL accordingly, was a possible virulence factor related with necrotic lesions of the skin and subcutaneous tissues like furuncles. Moreover, antimicrobial activity using eight antibiotics was tested by

disk diffusion assay. As such, oxacillin and oxytetracycline were the least effective antibiotics, while vancomycin and linezolid constituted the most successful drugs to treat staphylococcal infections. Besides the difference in antimicrobial susceptibility patterns, PVL negative and positive *S. aureus* isolates were also found to be different on the molecular, microbiological and epidemiological levels. Furthermore, the majority of PVL negative isolates exhibit white small colonies, as opposed to the majority of PVL positive isolates that appear orange large colonies when plated on mannitol salt agar.

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To the end that my glory
may sing praise to thee,
and not be silent. O Lord
my God, I will give thanks
unto thee for ever.
— *Psalms 30.12*

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This thesis is dedicated to my precious father and loving mother.

LIST OF ABBREVIATIONS

PVL: Panton-Valentine Leukocidin

PMN: Polymorphonuclear Leucocytes

SCC*mec* : Staphylococcal Cassette Chromosome *mec*

att site: attachement site

SLT: Staphylococcal Leukocytolytic Toxin

TSST: Toxic Shock Syndrome Toxin

MSCRAMM: Microbial Surface components Recognizing Adhesive Matrix Molecules

SCV: Small Variant Colonies

PIA: Polysaccharide Intercellular Adhesin

Sag: Superantigen

ORF: Open Reading Frame

CA-MRSA: Community-Acquired Methicillin-resistant *Staphylococcus aureus*

HA-MRSA: Hospital-Acquired Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin sensitive *Staphylococcus aureus*

agr: Accessory Gene Regulator

IL-6: Interleukin-6

CD14: Cluster of Differentiation 14

SE: Staphylococcal Enterotoxins

ET: Exfoliative Toxins

PTSAgs : Pyrogenic Toxin Superantigens

MHC: Major Histocompatibility complex

SFP: Staphylococcal Food Poisoning

TSS: Staphylococcal Toxic Shock Syndrome

SSSS: Staphylococcal Scalded Skin Syndrome

INTRODUCTION

Staphylococcus aureus, the most virulent *Staphylococcus* species, is considered as the most prevailing pathogen isolated from patients in hospitals and the second most widespread in patients in outpatient settings (Boyle-Vavra & Daum, 2007). *S. aureus* causes a wide range of diseases from mild superficial skin infection to life-threatening bacteraemia and infective endocarditis as well as toxinoses such as toxic shock syndrome (Johnsson et al., 2004). The lethality rate of such infections ranges from 30% to 80% (Lopez-Aguilar et al., 2007). *S. aureus* is known to be a difficult organism to treat because of its resistance to various antibiotics, mainly methicillin, a β-lactam antibiotic. Methicillin-resistance is encoded by the *mecA* located within the staphylococcal cassette chromosome *mec* (SCC*mec*) (Vandenesh et al., 2003 ; Francois et al., 2004).

The pathogenicity of *S. aureus* infections is correlated with various surface components and extracellular proteins including released hemolysins, enterotoxins exfoliatins, toxic shock syndrome toxin and Panton-Valentine Leukocidin (PVL) (Lina et al., 1999).

The γ -toxin is expressed by 99% of *S.aureus* strains (Prevost et al., 1995 ; Nilsson et al., 1999 ; Dinges et al., 2000 ; Johnsson et al., 2004). The *hlg* locus can express two functional pairs of proteins, *hlgA+hlgB* and *hlgC+hlgB*, both effectively display erythrocytes hemolysis isolated from human and other mammalian species (Kamio et al., 1993 ; Lina et al., 1999 ; Nilsson et al., 1999 ; Kaneko & Kamio, 2004 ; Johnsson et al., 2004) by inserting in the lipid bilayer (Viero et al., 2006). They are also capable of lysing human leukocytes and are cytotoxic to human lymphoblast cells (Rogolsky, 1979 ; Lina et al., 1999).

On the other hand, PVL is cytotoxic to human and rabbit monocytes, macrophages and polymorphonuclear leukocytes (PMN), without any hemolytic activity (Panton & Valentine, 1932 ; Prevost et al., 1995 ; Morinaga et al., 2003 ; Viero et al., 2006). It is also dermonecrotic and cytolytic and is associated with disease states that range from superficial skin infections to severe septicemia (Bhakdi & Tranum-Jensen, 1991 ; Gouax et al., 1997 ; Prevost et al., 1995 ; Lina et al., 1999 ; Kaneko & Kamio, 2004 ; Said-Salim et al., 2005 ; Boyle-Vavra & Daum, 2007). PVL has been detected in less that 5% of *S. aureus* isolates causing necrotic lesion, involving the skin, and severe necrotizing pneumonia (Lina et al., 1999 ; Gillet et al., 2000 ; An Diep et al., 2004 ; Francis et al., 2004 ; Johnsson et al., 2004 ; Moroney et al., 2006 ; Labandeira et al., 2007). Unlike hospital-acquired methicillin resistant *S. aureus* (HA-MRSA), most community-acquired MRSA (CA-MRSA) strains express the highly potent transferable toxin locus, PVL (Liassine et al., 2004; Moroney et al., 2006 ; Labandeira et al., 2007). Added to that, in some locations, the PVL locus is more common among CA-MRSA isolates than CA-methicillin sensitive *S. aureus* (CA-MSSA) isolates suggesting that this combination of PVL with the *mecA* gene has created a superadapted "ecologically fitted" *S. aureus* strain that is spreading in the community, maybe due to their short doubling time (Dufour et al., 2002 ; Boyle-Vavra & Daum, 2007 ; Labandeira et al., 2007 ; Maree et al.,2007). PVL accordingly, is considered as a marker of virulence (Panton & Valentine, 1932 ; Prevost et al., 1995 ; Lina et al., 1999 and Boyle-Vavra & Daum, 2007),

The present study aims at the use of a duplex PCR in order to obtain insights into the prevalence of PVL and γ-hemolysin genes and determines the antimicrobial activity of eight different antibiotics using the disk-diffusion assay, within *S. aureus* clinical isolates isolated from major tertiary care medical centers in Lebanon. This study additionally aims at

determining whether the PVL gene represents a stable marker of the CA-MRSA strains and a marker of virulence in Lebanon.

Concerning Lebanon, there is a lack of epidemiologic data. To the extent of our knowledge, our study is unique in Lebanon since it is the only one that targets the prevalence of PVL and γ-hemolysin genes in *S. aureus* strains. Added to that, this study is of great importance for Lebanon because the samples tested are believed to be representatives of the population of *S. aureus* in Lebanon. Patients from the various Lebanese cities and rural areas tend to frequent the three hospitals from where samples were collected, since they are considered as major tertiary care centers in the country.

Previous studies conducted in Lebanon explored solely the antibiotic resistance patterns of *S. aureus* such as with Araj et al. (1994), Kanj et al. (2004) and Borg et al. (2006). Moreover, the prevalence of PVL and γ-hemolysin genes has not been studied in the Middle East.

LITTERATURE REVIEW

2.1. Overview of *Staphylococcus aureus*

In 1882, the Scottish surgeon Sir Alexander Ogston named the organism "*Staphylococcus*" which is now recognized as an extremely successful human pathogen (Francis et al., 2004). Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes, found as bacterial pathogens and commensal organisms in both humans and animals (Piano, 2003; Todar, 2006). They are non motile, non spore-forming and catalase positive (Tolan & Boarto, 2006), able to convert hydrogen peroxide (H_2O_2) to water and oxygen making the catalase test useful to distinguish staphylococci from enterococci and streptococci (Todar, 2006). These organisms are resistant to harsh conditions like temperatures as high as 50°C, high salt concentrations and drying (Tolan & Boarto, 2006) and can be recovered from non-physiologic environments up to months after inoculation (Piano, 2003). They can grow easily under numerous conditions in the presence of oxygen (Piano, 2003), and can also grow anaerobically yielding principally lactic acid (Todar, 2006). Taxonomically, the genus *Staphylococcus* belongs to the Bacterial family *Staphylococcaceae*, that includes three lesser known genera, *Gamella*, *Macrococcus* and *Salinicoccus*. The best-known of its nearby phylogenetic relatives are the members of the genus *Bacillus* in the family *Bacillaceae*, which is on the same level as the family *Staphylococcaceae* (Todar, 2006).

Of the staphylococci, *Staphylococcus aureus* is the most significant pathogen causing both human and animal diseases (Piano, 2003). It has about 2,600 genes and 2.8 million bp of DNA in its chromosome. It is a frequent

component of the human microbial flora that can turn into a dangerous pathogen. As such, this organism is capable of infecting almost every tissue and organ system in the human body. It does so by exporting a variety of virulence factors to the cell surface and extracellular milieu of the human host (Todar, 2006). A traditional marker for the identification of *S. aureus* in the clinical microbiology laboratory is the coagulase test (Bhakdi et al, 1991 ; Piano, 2003). Coagulase is an extracellular protein, which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin (Todar, 2006). It is also a virulence factor since clotting induced by coagulase results in the accumulation of fibrin around the bacterial cells making it difficult for the host defense agents to come into contact with the bacteria and making the staphylococci resistant to phagocytosis (Madigan et al., 2003). Its production is negatively regulated by *agr* (Fischetti et al., 2000). There was a confusion in the literature concerning the coagulase and the clumping factor which is a fibrinogen-binding determinant on the *S. aureus* cell surface (Todar, 2006). A fraction of coagulase which is associated with the bacterial cell surface, was therefore thought to be responsible for the clumping of bacterial cells when mixed with plasma (Fischetti et al., 2000). However, clumping factor is distinct fibrinogen-binding protein that can promote binding of bacteria to solid-phase fibrinogen in contrast to coagulase which binds only soluble fibrinogen (Fischetti et al., 2000).

2.2. Pathogenesis of *Staphylococcus aureus*

S. aureus is a very successful hospital and community-acquired pathogen (Holmes, 2005 ; McDonald et al., 2005); it is the most prevalent pathogen isolated from hospitalized patients and the second most common from patients in outpatient settings (Boyle-Vavra & Daum, 2007). It is carried by

30 to 40% of the population. Approximately 35% of the general populations are commensal nasal carriers and most newborns will be colonized within the first week of life. (Dancer & Noble, 1991 ; Piano, 2003). It can be identified readily in the nose, but the organism can also be detected in other moist regions of the human body, such as the axillae, perineum, vagina, and rectum, which thereby form a major reservoir for infections (Todar, 2006). It causes a variety of diseases ranging from mild superficial skin infection to life threatening bactaeremia and infective endocarditis, as well as toxin-mediated conditions such as toxic shock syndrome (Johnsson et al., 2004). A variety of predisposing factors may lead to more serious infections such as conjunctivitis, osteomyelitis, septicemia, empyema, septic arthritis, meningitis, pneumonia, pericarditis or endocarditis (Piano, 2003; Holmes 2005). The lethality rate of such infections ranges from 30% to 80% (Lopez-Aguilar et al., 2007). Diseases caused by *S. aureus* can be the result of direct tissue invasion or due to the action of a variety of exotoxins released by the bacteria (Iandolo, 1989 ; Marrack and Kappler, 1990 ; Piano, 2003). The risk of intravascular and systemic infection by *S. aureus* rises when the epithelial barrier is disrupted by intravascular catheters, implants, mucosal damage, or trauma. Interestingly, after infection, cells of *S. aureus* can persist unnoticed in the human body for a long time (years), after which they can suddenly cause another infection. *S. aureus* is primarily an extracellular pathogen whose colonization and invasion of human tissues and organs can lead to severe cytotoxic effects. Nevertheless, *S. aureus* can also be internalized by various cells, including nonphagocytic cells, which seems to induce apoptosis (Todar, 2006).

Pathogenicity is related to a number of virulence factors that allow it to adhere to surfaces, invade or avoid the immune system and cause harmful toxic effects to the host (Holmes 2005). These virulence factors include: surface proteins that promote adhesion to and colonization of host tissues

(Piano, 2003 ; Sibbald et al., 2006), invasins that are exported to an extracytoplasmic location and promote bacterial spread in tissues (leukocidin, kinases, and hyaluronidase) (Sibbald et al., 2006), surface factors that inhibit phagocytic engulfment (capsule and protein A) (Lina et al., 1999 ; Holmes et al., 2005 ; Sibbald et al., 2006), biochemical properties that enhance staphylococcal survival in phagocytes (carotenoid and catalase production) (Chamberlain et al., 1991; Liu et al., 2005; Sibbald et al., 2006 ; Besier et al., 2007), immunological disguises (protein A, coagulase, and clotting factor) (Sibbald et al., 2006), membrane-damaging toxins that disrupt eukaryotic cell membranes (hemolysins and leukotoxin) (Cooney et al., 1993; Nilsson et al., 1999; Kaneko et al., 2004 ; Sibbald et al., 2006), superantigens that contribute to the symptoms of septic shock (SEA-G, toxic shock syndrome toxin [TSST], and ET) (Clyne et al, 1988; Piano, 2004 ; Sibbald et al., 2006) and determinants for inherent and acquired resistance to antimicrobial agents (Centers for Disease Control and Prevention, 1997; 2000a; 2000b ; Piano, 2004 ; Sibbald et al., 2006).

2.3. Methicillin resistance of *Staphylococcus aureus*

The resistance to methicillin emerged directly after the introduction of the drug into clinical use. It played an important factor that helped *S. aureus* to establish nosocomial infections (Francis et al., 2004 ; Voyich et al., 2006). Some methicillin-resistant *S. aureus* (MRSA) strains spread easily and are known as epidemic MRSA, while strains lacking this capacity are associated with sporadic cases (Zee et al. 2005). Worldwide infections caused by community-acquired (CA)-methicillin-resistant *S. aureus* were reported (Vandenesch et al. 2003). Methicillin resistance in *S. aureus* is mediated by the production of low-affinity penicillin binding protein 2a that is encoded by the *mecA* gene (Kilic et al., 2006). This *mecA* gene is located on a mobile genetic element designated as staphylococcal cassette chromosome *mec* (*SCCmec*) (Francis et al., 2004). Sequence analyses defined

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three major *SCCmec* types (*SCCmec* types I, II and III) among nosocomial MRSA strains (Said-Salim et al., 2005). Type IV and V were described to be associated with community-acquired MRSA (Ca-MRSA) throughout the world (Vandenesch et al., 2003 ; Kilic et al., 2006). CA-MRSA strains differ from nosocomial MRSA strains or hospital-acquired MRSA strains (HA-MRSA) on the basis of their genetic backgrounds and antibiograms (Okuma et al., 2002 ; Said-Salim et al., 2005). *SCCmec* type IV element is characterized by having a small size, lack of antibiotic resistance markers and the presence of functional recombinases. That's why CA-MRSA are found to be more susceptible to antibiotics than HA-MRSA. Another characteristic that distinguishes CA-MRSA strains from the multidrug resistant HA-MRSA is that they show resistance only to β-lactam antibiotics (Said-Salim et al., 2005) and carry the genes for Panton-Valentine Leukocidin (PVL), a bicomponent cytotoxic virulence factor associated with skin and soft tissue infections as well as with more serious infections like severe necrotizing pneumonia (An Diep et al., 2004 ; Voyich et al., 2006 ; Labandeira et al., 2007).

2.4. *Staphylococcus aureus* Carotenoids

Subsequent studies on *S. aureus* unraveled the cause of their golden hue appearance by the presence of an elaborate biosynthetic pathway that yields a series of colored pigments known as carotenoids (Liu et al., 2005). Carotenoids are isoprenoid lipids containing 40 carbon atoms (eight isoprene units) and are present in the cell membranes of many microorganisms, as well as in dietary fruits and vegetables (Hammond & White, 1970 ; Liu et al., 2005). The degree of unsaturation and conjugation of double bonds ranges between colorless saturated polyenes to conjugated, hydroxylated carotenoids which are intensely colored (Hammond & White, 1970 ; Marshall & Wilmoth, 1981). At the stationary phase, over 90% of *S. aureus* colonies appear deeply orange.

Staphyloxanthin, a glycosylated carotenoid and its pigmented precursors are lipophilic and embedded in the cell membrane (Chamberlain et al., 1991). Production of Staphyloxanthin is recognized as potent antioxidant that resists oxidant-based clearance mechanisms of the innate immune response by virtue of its free-radical scavenging properties and exceptional ability to quench singlet oxygen (Liu et al., 2005). Added to that, it plays an important role in the membrane stabilization and in the prevention of potentially lethal fatty acid-induced changes in the membrane fluidity (Chamberlain et al., 1991). Thus, carotenoids are considered as key virulence factor that protect *S. aureus* from the phospholipases present in macrophages and polymorphonuclear neutrophils that hydrolyze phospholipids to produce fatty acids. Fatty acids form bactericidal agents that incorporate in the *S. aureus* membrane increasing the membrane fluidity and decreasing the vital membrane-associated functions (Chamberlain et al., 1991). However, some *S. aureus* strains manifest fastidious growth characteristics with reduced hemolysin production and increased intracellular survival. These subpopulations are designated as "small variant colonies" (SCVs) (Sifri et al., 2006). In contrast to the normal *S. aureus* phenotype, SCVs grow as tiny, nonpigmented and nonhemolytic colonies. They are characterized by their altered expression of virulence, auxotrophism for distinct growth factors such as thymidine, hemin and/or menadione (Besier et al., 2007), increased susceptibility to singlet oxygen killing and decreased ability to survive in the murine whole blood (Liu et al., 2005), and a notorious resistance to aminoglycoside antibiotics (Fischetti et al., 2000). Intracellular location shields *S. aureus* from host defenses and limit exposure to certain antibiotics, thus it is thought to be a critical feature of the ability of SCVs to cause chronic and persistent infections (Sifri et al., 2006). This explains the isolation of SCVs from osteomyelitis, persistent skin and wound infection, device-related infections and cystic fibrosis lung disease (Besier et al., 2007).

2.5. Staphylococcal biofilms formation

S. aureus has the potential to form biofilms on indwelling catheters and other implanted devices. Two stages of staphylococcal biofilm formation have been described. The first stage involves attachment of cells to a surface. This stage of biofilm formation is likely to be mediated in part by cell wall–associated adhesins, including the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The second stage of biofilm development however, includes cell multiplication and formation of a mature, multi-layered, structured community. This stage is associated with production of extracellular factors, including the polysaccharide intercellular adhesin (PIA) component of the extracellular matrix. Detachment of cells from the established biofilm may then allow staphylococci to spread and colonize new sites (Yarwood & Schlievert, 2003)

2.6. *Staphylococcus aureus* exoproteins

S. aureus produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in mammalian hosts (Dinges et al, 2000). The exotoxins fall into 2 groups: membrane-active agents and toxins with superantigen (Sag) activity (Fishetti et al, 1994). Nearly all strains secrete a group of enzymes and cytotoxins which includes six cytolytic toxins alpha, beta, gamma and delta-hemolysins, leukocidin (*Luk*) and Panton-Valentine Leukocidin (PVL)(Kaneko et al., 2004), nucleases, proteases, lipases, hyaluronidase, and collagenase (Dinges et al., 2000). The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth (Dinges et al, 2000).

2.6.1. Hemolyins and Leukocidins

2.6.1.1. γ -Hemolysin

Two types of bicomponent toxins are made by *S. aureus*, Gamma-Hemolysin and Panton-Valentine (PV) leukocidin (Dinges et al., 2000 ; Viero et al., 2006). The gamma-toxin locus is expressed by 99% of *S.aureus* strains (Prevost et al., 1995 ; Nilsson et al., 1999 ; Dinges et al., 2000 ; Johnsson et al., 2004). The 3,797 bp sequence of the *hlg* locus includes three open reading frames, designated as *hlgA*, *hlgB* and *hlgC* (Cooney et al., 1993). These three proteins are classified as: two class S (Slow-eluted) component comprising *hlgA* and *hlgC* and one class F (Fast-eluted) component which is *hlgB* (Nilsson et al., 1999). Thus the *hlg* locus can express two functional pairs of proteins, *hlgA+hlgB* and *hlgC+hlgB*, both effectively display erythrocytes hemolysis isolated from human and other mammalian species (Kamio et al., 1993 ; Lina et al., 1999 ; Nilsson et al., 1999 ; Kaneko & Kamio, 2004 ; Johnsson et al., 2004) by inserting in the lipid bilayer (Viero et al., 2006). They are also capable of lysing human leukocytes and are cytotoxic to human lymphoblast cells (Rogolsky, 1979 ; Lina et al., 1999). The putative ribosome binding site lies within an open reading frame, designated *hlgC*, that is upstream from *hlgB*. The *hlgC* gene has a potential ribosome binding site and also a promoter. It is likely that these two genes are cotranscribed while the *hlgA* gene spans a region defined by several transposon insertions. A putative promoter and a ribosome binding site are located 5' to the coding sequence, and a transcriptional termination sequence occurs 3' to the coding sequence. Thus, *hlgA* is more likely to be monocistronic (Cooney et al., 1993 ; Dinges et al., 2000). *hlgA* specifies γ1 component of the gamma toxin since a sequence of amino acids starting at residue 30 of the translation product of *hlgA* corresponds exactly to the amino terminal sequence of γ1 (Cooney et al., 1993). *hlgA* (γ1) and *hlgC* (γ2), sharing 70% of residue identity (Cooney et al., 1993), assemble into a ring shaped 195-kDa complex in a molar ratio

of 1:1 on the human erythrocytes membrane, forming a transmembrane pore with a functional diameter of 2.1-2.4 nm (Yokota et al., 1998). Thus gamma-hemolysin is referred to as pore forming toxins (PFTs) that belong to the transmembrane β-barrel family (Nguyen & Kamio, 2004). *hlgA* and *hlgB* bind to membranes as monomers (Fig.1) (Viero et al., 2006).
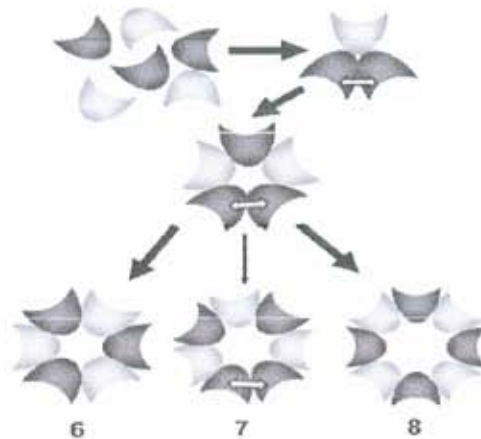
Fig.1: Topology and assembly pathway of the *hlgA* and *hlgB* pore (Viero et al., 2006)

Until now, the stoichiometry of the leucotoxin pore has been controversial, with heptamers suggested by electron microscopy (Tomita et al., 2002 ; Viero et al., 2006), or octamers identified by the analysis of labelled or covalently linked proteins (Miles et al., 2002 ; Viero et al., 2006) but hexamers were also proposed (Ferreras et al., 1998 ; Viero et al., 2006) (Fig.4). However, after extensive electron microscope and biochemical analysis, Kaneko and Kamio (2004) found that the gamma-hemolysin assembly occurs in a stochastic manner to form alternate complexes with subunit stochiometries of 3:4 and 4:3. In order to monitor the *hlg*-induced hemolysis, gamma-hemolysin was incubated with intact erythrocytes for 10min. Intact disc-shaped erythrocytes became swollen, round shaped cells with clear edges. Soon after, the swollen cells lysed. Usually cells swelling is caused by permeabilization of cell membranes , thus *hlg* induced colloid

osmotic lysis of human erythrocytes through pore formation (Kaneko & Kamio, 2004). The number of toxin molecules per cell and the pore diameter are affected by divalent cations, especially Ca^{2+}, thus resulting in varying erythrocytes susceptibility responses (Fischetti et al., 2000). As the concentration of the toxin increases, *hlg* pores have a great tendency to condense into clusters. The aggregation of pores is not only driven by the distribution of their membrane receptors, but also by noncovalent linkages between amino acids present at the outer surfaces of the pores. This pore aggregation offers two advantages; first the switching of the equilibrium balance of single pore assembly toward association, thereby increasing the total number of pore per cell. Another advantage of the large cluster of pores on the membrane is that it weakens the cell membranes and enhances cell bursting (Kaneko & Kamio, 2004). Sugawara et al. (1997) has noticed that cell membranes seem to be disrupted in areas surrounding the clusters.

2.6.1.2. Panton-Valentine Leukocidin (PVL)

S. aureus produces several leukotoxins, the most important is Panton-Valentine Leukocidin (Morinaga et al., 2003 ; Viero et al., 2006). Panton and Valentine in 1932 were the first to have reported the presence of a leukocidal activity in a V8 strain, that is cytotoxic to human and rabbit monocytes, macrophages and polymorphonuclear leukocytes (PMN), without any hemolytic activity (Panton & Valentine, 1932 ; Prevost et al., 1995 ; Morinaga et al., 2003 ; Viero et al., 2006). Panton-Valentine Leukocidin has also dermonecrotic and cytolytic properties and it is associated with disease states that range from superficial skin infections to severe septicemia (Bhakdi & Tranum-Jensen, 1991 ; Gouax et al., 1997 ; Prevost et al., 1995 ; Lina et al., 1999 ; Kaneko & Kamio, 2004 ; Said-Salim et al., 2005 ; Boyle-Vavra & Daum, 2007). However, unlike other *S. aureus* pore-forming leukocidins, PVL is not hemolytic (Boyle-Vavra & Daum,

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2007). PVL has been detected in less that 5% of *S. aureus* isolates causing necrotic lesion, involving the skin, and severe necrotizing pneumonia (Lina et al., 1999 ; Gillet et al., 2000 ; An Diep et al., 2004 ; Francis et al., 2004 ; Johnsson et al., 2004 ; Moroney et al., 2006 ; Labandeira et al., 2007). According to Johnsson et al. (2004), 93% of the PVL-positive isolates are associated with furunculosis, 55% with cellulitis, 50% with cutaneous abscess and 13% with finger-pulp infection (Johnsson et al., 2004). The majority (85%) of the highly lethal necrotizing pneumonia cases were caused by PVL-positive *S. aureus* found in young healthy patients (Francis et al., 2004). Unlike HA-MRSA, most CA-MRSA strains express the highly potent transferable toxin locus, PVL (Liassine et al., 2004; Moroney et al., 2006 ; Labandeira et al., 2007). Added to that, in some locations, the PVL locus is more common among CA-MRSA isolates than CA-MSSA isolates suggesting that the methicillin resistance has contributed to the success of PVL-positive *S. aureus* strains (Boyle-Vavra & Daum, 2007 ; Labandeira et al., 2007). However, PVL is less often present in CA-MRSA isolates associated with asymptomatic nasal colonization (Boyle-Vavra & Daum, 2007). Boubaker et al. (2004) reported that PVL-positive *S. aureus* may spread between persons in close contact, with the initial manifestations being in the form of skin infections progressing to severe necrotizing pneumonia with a high death rate. Hence, PVL is considered as a marker of virulence (Panton & Valentine, 1932 ; Prevost et al., 1995 ; Lina et al., 1999 ; Boyle-Vavra & Daum, 2007). The protein forms nonspecific pores in the leukocyte plasma membrane which result in increased permeability and eventual host cell lysis (Said-Salim et al., 2005). This bi-component pore-forming exotoxin is encoded by two contiguous co-transcribed genes, *lukS-PV* and *lukF-PV*, carried on a prophage integrated in the *S. aureus* chromosome (Prevost et al., 1995 ; Morinaga et al., 2003 ; Denis et al., 2005 ; Said-Salim et al., 2005). Two types of prophages have been found to carry this toxin: ΦSLT (Staphylococcal Leukocytolytic Toxin)

(Kaneko & Kamio, 2004 ; McDonald et al., 2005) and ΦPVL (Kaneko & Kamio, 2004). The genome of the PVL-carrying phage ΦPVL is 41,402 bp long and contains 62 ORFs. It is made by lysogeny, replication, packaging and the regions coding for head, tail and lysis. The PVL genes are located next to the *att* (attachment) site. The structure of the ΦSLT is different from that of ΦPVL. It has an elongated head and a flexible tail, while ΦPVL has an isometric hexagonal head. The genome of ΦSLT is 42,942 bp long, made up of 62 ORFs. ΦSLT and ΦPVL are similar in that they both have the same *att* sites, the same site of integration in the host chromosomal DNA and the same PVL genes. However, some differences exist between the two phages in other regions. For example, the N terminal of ΦPVL is 116 amino acids, with a highly coiled structure of precapsid protein that needs to be removed to become mature, while the N-terminal of ΦSLT is 113 amino acids long forming a mature capsid protein (Kaneko & Kamio, 2004).

The mechanism by which PVL-positive CA-MRSA emerged suggests that a methicillin sensitive *S. aureus* (MSSA) strain is infected by the lysogenic phage ΦSLT. At the same time a methicillin resistance cassette (SCCmec IV or V) carrying the *mecA* gene is horizontally transferred into the PVL positive MSSA strain and integrates into the genome in a location that is distinct from that of the ΦSLT integration site. A few lineages endowed with the best fitness traits have survived. Indeed, the faster growth rates of CA-MRSA suggest that they are more fit than HA-MRSA (Fig. 2) (Boyle-Vavra & Daum, 2007).
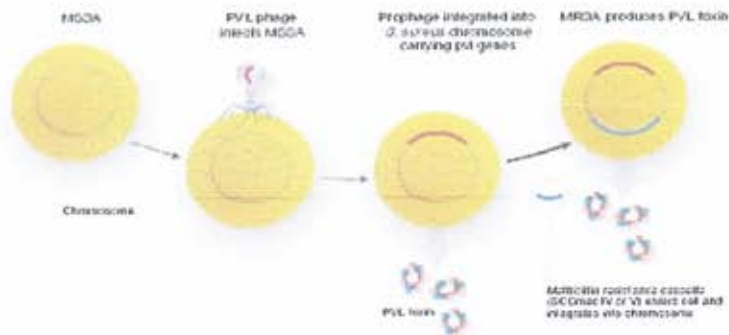
Fig. 2: Model for the emergence of PVL producing CA-MRSA

lukS-PV and *lukF-PV* are separated by a single thymine nucleotide and transcribed as a single mRNA molecule. The *lukS-PV* is 939 nucleotides in size and *lukF-PV* 978 nucleotides. *lukS-PV* (slow-eluted) and *lukF-PV* (fast-eluted) are classified accordingly on the basis of their separation by column chromatography (Prevost et al., 1995, Morinaga et al., 2003 ; Johnsson et al., 2004 ; Labandeira et al., 2007). Neither F nor S component alone has cytotoxic activity, but both together are active (Morinaga et al., 2003). PVL and γ -Hemolysin form a bi-component toxin. They belong to the same toxin family known as synergohymenotropic toxins that act on the cell membranes, in order to form pores, by the synergy of two proteins (Fig. 3) (Prevost et al., 1995 ; Lina et al., 1999).

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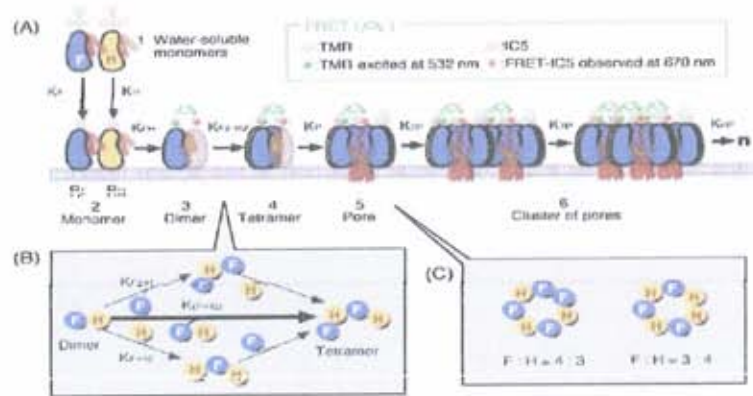
Fig. 3: Schematic model of assembly of *lukF* and hlg2 for hetero-oligomeric pore formation. (A) Possible process of tetramerization of *hlg* oligomers and (B&C) heptameric pores of alternate complexes with subunit stoichiometries of 3:4 and 4:3 (Kaneko & Kamio, 2004).

S-component of the toxin binds first to the membrane and then the consecutive interaction of F-component induces hetero oligomers (Morinaga et al., 2003). Kaneko & Kamio (2004) found out that phosphorylation of *lukS* is crucial for the *lukS*-specific function of Luk toxin. *lukS-PV* initiates binding to an unidentified receptor on PMN leukocytes membrane where a dimerization with *lukF-PV* takes place followed by an alternate serial binding of *lukS-PV* and *lukF-PV* until the heptamer is assembled (Boyle-Vavra & Daum, 2007). *lukS-PV* phosphorylation by protein kinase A or C takes place on the human PMN leukocytes membrane after its initial binding followed by the induction of Ca^{++} ion channels, thus regulating ion such as calcium current through the membrane (Kaneko & Kamio, 2004 ; Boyle-Vavra & Daum, 2007). Then, the subsequent binding of *lukF* is needed to induce cell lysis (Kaneko & Kamio, 2004). Hence, the induction of signal transduction events triggers the production of interleukins and inflammatory mediators (Fig. 4) (Boyle-Vavra & Daum, 2007).
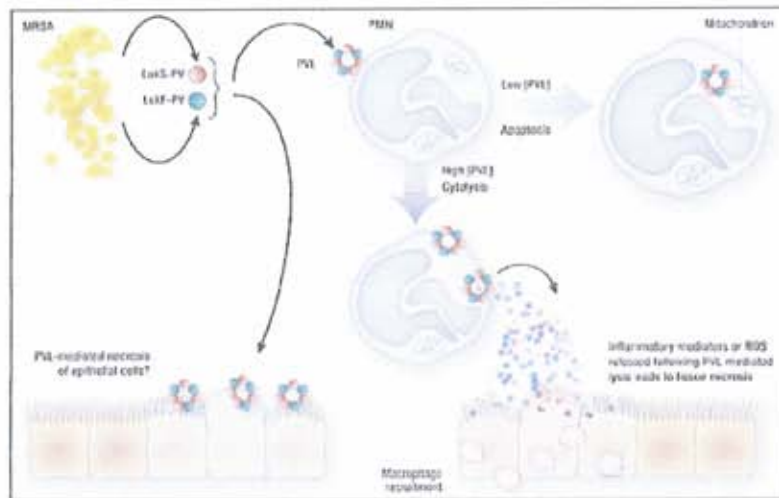
Fig. 4: Model for how PVL might mediate tissue necrosis (Boyle-Vavra & Daum, 2007).

Another leukocytolytic effect of *lukS-PV* is concluded due to the fragility of human PMN leukocytes that is caused by the induction of a lytic enzyme, such as an autolytic enzyme or a phospholipase A2 that degrades the membrane (Kaneko & Kamio, 2004). Depending on the concentration of PVL, it can cause either PMN lysis, leading to neutropenia, or apoptosis. The latter occurs via a novel pathway that presumably involves PVL-mediated pore formation in the mitochondrial membrane leading to a release of cytochrome *c* and induction of caspases 9 and 3 (Fig. 5) (Boyle-Vavra & Daum, 2007).
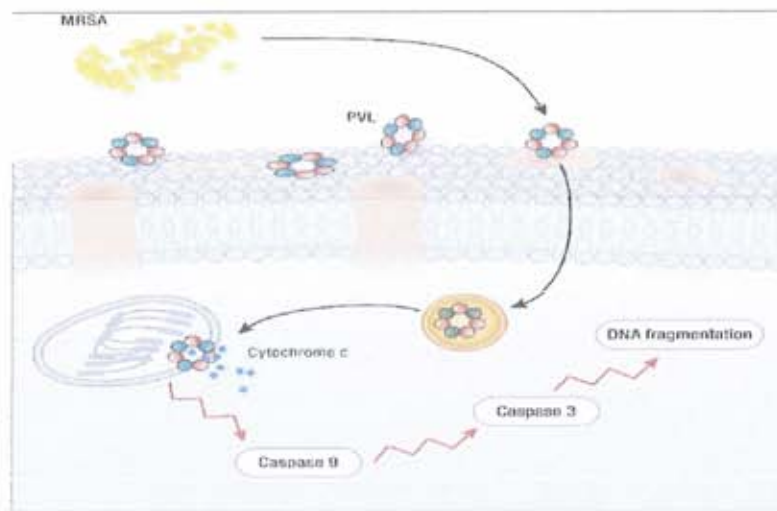
Fig. 5: Unique mechanism of PVL-induced PMN apoptosis (Boyle-Vavra & Daum, 2007)

PVL-mediated PMN lysis and apoptosis constitute the first step in the evasion of the first line of host immune defense. However, PVL is not directly involved in tissue necrosis. It indirectly mediates tissue necrosis and sepsis by either the release of cytotoxic lysosomal granule contents from lysed PMNs or by an inflammatory cascade set in motion by PMN lysis or apoptosis. This, in turn, induces the release of reactive oxygen species and a variety of inflammatory mediators from granulocytes (Fig. 4) (Boyle-Vavra & Daum, 2007).

2.6.1.3. α-Hemolysin

Alpha toxin has always been considered to play a significant role in the pathogenesis of staphylococci (Rogolsky, 1979). Its main significance in pathogenicity is that of producing tissue damage after establishing a focus of infection (O'Callaghan et al., 1997). The toxin gene *hla* is present in a single copy in the bacterial chromosome, and has the accessory gene regulator (*agr*) a transactive positive control element (Bhakdi & Tranum-Jensen, 1991 ; O'Callaghan et al., 1997 ; Dinges et al., 2000). Aside from

being hemolytic, Alpha-toxin is a membrane damaging toxin or cytolytic pore-forming toxin (Rogolsky, 1979 ; Fischetti et al, 2000). It is also dermonecrotic and neurotoxic (Dinges et al, 2000). Erythrocytes, mononuclear immune cells, epithelial and endothelial cells, Hela cells, Ehrlich ascites carcinoma cells and platelets isolated from different species show different patterns of susceptibilities to the toxin (Rogolsky, 1979 ; Bhakdi & Tranum-Jensen, 1991 ; Fischetti et al., 2000). It has been observed that binding can occur in two different ways: when alpha-toxin is applied at high concentrations, binding occurs probably via the intrinsically inefficient absorptive interaction (Bhakdi & Tranum-Jensen, 1991); it adheres to the cell membrane, probably through a membrane protein (Dinges et al, 2000 ; Fischetti et al, 2000). However, when it is applied at low concentrations, binding is limited to very small number of specific sites that are expressed by certain cells only (Bhakdi & Tranum-Jensen, 1991). Alpha-toxin binds in monomer form to target cells membrane (Nguyen & Kamio, 2004), where they form cylindrical heptamers (Dinges et al, 2000). This heptamerization occurs upon their subsequent collision during lateral diffusion in the bilayer (Bhakdi & Tranum-Jensen, 1991). Once the cylindrical heptamer has formed in the cell membrane, a 1- to 2-nm pore is formed (Fig. 6). This pore forms via a stepwise process (Dinges et al., 2000).
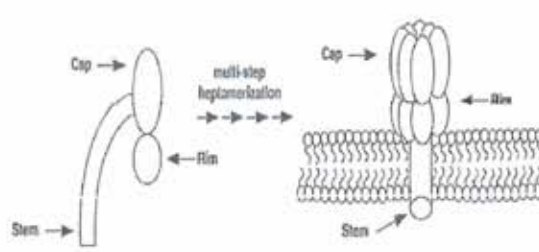
Fig. 6: Heptamerization of alpha-toxin and pore formation (Dinges et al., 2000)

The toxin has a wide number of effects on the host largely due to the formation of unregulated pores for ion transmission across the membranes of a variety of cell types. Examples include the formation of pores in the membranes of endothelial cells, resulting in arachidonic acid metabolism due to Ca^{++} influx and the formation of thromboxane and prostacyclin that results from activation of this metabolic pathway, leading to vasoconstriction (Dinges et al, 2000). Additionally, alpha-toxin effects on platelets can lead to release of procoagulation factors via Ca^{++} influx and osmotic swelling causes a breakdown in cell integrity, with the effect of increasing vascular permeability (Dinges et al, 2000). Cell death occurs as a result of rapid egress of vital molecules such as ATP and the loss of the characteristic "milieu interieur" that is required to sustain metabolic processes (Bhakdi & Tranum-Jensen, 1991).

2.6.1.4. β-Hemolysin

Beta-toxin is a neutral sphingomyelinase C (Fischetti et al, 2000). It shares the distinction with alpha toxin of *Clostridium perfrigens* type A in being the only membrane damaging toxin (Rogolsky, 1979). Beta-hemolysin is produced in large quantity by a number of *S. aureus* strains, particularly animal isolates (Dinges et al, 2000). It is encoded by *hlb* gene which is disrupted, in lysogens, by prophage integration (Fischetti et al, 2000). It is

leukotoxic to a variety of cells and stimulates the release of interleukin 1B, IL-6 receptor and soluble CD14 from human monocytes, resulting in the inhibition of monocyte migration (Fischetti et al, 2000). Beta-toxin is also unique in its ability to induce hot-cold lysis. Incubation of sensitive erythrocytes with toxin at 37°C results in little or no lysis. However, lysis ensues if treated erythrocytes are chilled below 10°C (Rogolsky, 1979 ; Fischetti et al., 2000). The degree of the cell sensitivity depends on membrane sphingomyelin content (Fischetti et al, 2000). Thus corneal and scleral epithelial membranes form tissue target for beta-toxin during an ocular infection (O'Callaghan et al., 1997), since they have high sphingomyelin content (Broekhuyse, 1975). Beta-Hemolysin acts as a type C phosphatase, hydrolyzing sphingomyelin to phosphorylcholine and ceramide (Fischetti et al, 2000). This activity requires the presence of Mg^{++} (Dinges et al., 2000), while Co^{++} and Mn^{++} can enhance activity (Fischetti et al, 2000). Unlike lesions induced by pore forming toxins, beta-toxin causes invaginations of selected regions of the membrane. Cohesive forces are sufficient to hold the ceramide in position in the membrane. A phase separation occurs with condensation of ceramide into pools and collapse of the bilayers (Fischetti et al, 2000). Other factors that affect cells susceptibility to beta-toxin include centrifugation, osmotic shock and rapid alteration of pH and NaCl concentration (Rogolsky, 1979).

2.6.1.5. δ-Hemolysin

Delta-Hemolysin is encoded by *hld* gene that plays an important regulatory role in the accessory gene regulator (*agr*) system (Dinges et al., 2000). This toxin, however, is secreted without a signal peptide; in fact it has been suggested that it can itself make an efficient signal peptide (Dinges et al., 2000). Delta-toxin is another cytolytic, dermonecrotic, lethal *S. aureus* toxin (Fischetti et al., 2000). It is unique because of its relative hydrophobic nature, thermostability, low degree of cellular specificity, inhibition by

phospholipids and high degree of surface activity (Rogolsky, 1979). Delta-hemolysin acts as a surfactant to disrupt the cell membrane (Rogolsky, 1979 ; Dinges et al., 2000). Cytoplasmic leakage and lysis of cells exposed to delta-toxin occur without a demonstrable lag, similar to treatment with Triton X-100 and other detergents. (Fischetti et al., 2000). It is thought that the toxin inserts at least partly into the lipid bilayer, disordering lipid chain dynamics. It has also been suggested that the mode of lysis involves the formation of channels in membranes composed of aggregates of six molecules of delta-toxin. (Fischetti et al., 2000)

2.6.1.6. Other Exoproteins and Diseases

Some strains produce one or more additional exoproteins, which include: toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI) and the exfoliative toxins (ETA and ETB) (Dinges et al, 2000). These toxins share a set of immunomodulatory activities as a result of their SAg function that promote the ability of most pyrogenic toxins to induce toxic shock syndrome (TSS) (Fischetti et al, 1994). Their primary function in vivo may be to inhibit host immune responses to *S. aureus*. TSST-1 and the staphylococcal enterotoxins are also known as pyrogenic toxin superantigens (PTSAgs) (Dinges et al, 2000). The PTSAgs are a group of exotoxins secreted by either *S. aureus* or *Streptococcus pyogenes* that have been grouped together because they share several important biological characteristics (Dinges et al, 2000). The family of PTSAgs presently includes: TSST-1, and most of the staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, and SEH) (Fischetti et al, 1994 ; Popa & Tervaert, 2003). Each of these exotoxins exhibits at least three biological properties: pyrogenicity, superantigenicity and the capacity to enhance the lethality of endotoxin in rabbits up to 100,000-fold (Dinges et al, 2000). Some PTSAgs possess additional properties. For example, the staphylococcal

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enterotoxins (SEs) are potent emetic agents whereas the other PTSAgs are not. Also, TSST-1 is unique in its ability to cross mucosal surfaces and it is the only PTSAg known to reactivate bacterial cell wall-induced arthritis. (Dinges et al, 2000) The best-characterized property of the PTSAgs is superantigenicity. Superantigens have the ability to bypass normal major histocompatibility complex (MHC) - restricted, intracellular, antigen processing and presentation. Superantigens (SAg) activate up to 50% of the whole T cell repertoire rather than the 1% fraction stimulated by conventional antigens, by direct binding to the MHC class II molecule and the T cell receptor, at sites away from those involved in conventional antigen binding (Llewelyn & Cohen, 2001).

The staphylococcal PTSAgs cause or have been implicated in the pathogenesis of several acute or chronic human disease states. Two human diseases that are definitely caused by PTSAgs elaborated by *S. aureus*: staphylococcal food poisoning (SFP) and staphylococcal toxic shock syndrome (TSS) (Dinges et al, 2000 ; Katoh et al., 1988).

SFP results from ingestion of one or more preformed SEs on food that has been contaminated with *S. aureus* (Dinges et al., 2000). Its symptoms include nausea, vomiting, retching, abdominal cramping and prostration (U.S. Food and Drug Administration, 1992). They are highly correlated with the generation of a number of inflammatory mediators, including prostaglandin E2, leukotriene B4, and 5-hydroxyeicosatetraenoic acid. SFP is a self-limited condition that typically resolves within 24 to 48 h of onset (Dinges et al., 2000). On the other hand, toxic shock syndrome toxin-1 (TSST-1) produced by phage group I strains of *S. aureus* causes toxic shock syndrome (TSS); a multisystem illness (Clyne et al., 1988). It is characterized by high fever, diffuse erythematous rash, desquamation of the skin 1 to 2 weeks after onset, hypotension, vomiting, diarrhea and occasionally death (Madigan et al., 2003; Dinges et al., 2000). TSS is considered a capillary leak syndrome that is manifested clinically as

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hypotension, hypoalbuminemia, and generalized nonpitting edema (Dinges et al., 2000). TSST-1 is released by the growing staphylococci, causing a massive T-cell reaction resulting in the inflammatory response characteristic of superantigen reactions (Madigan et al., 2003).

Added to that, *S. aureus* exfoliative toxins ETA and ETB are capable of causing an exfoliative dermatitis known as Staphylococcal Scalded Skin Syndrome (SSSS) (Piano, 2003). SSSS shares many features with other superantigen-mediated syndromes that include the erythematous rash, tachycardia, hypotension, fever and shock (Ladhani et al., 1999). It is characterized by formation of bullae or skin blisters and includes Ritter's disease, toxic epidermal necrosis, bullous impetigo and some cases of erythema (Fischetti et al, 2000). It involves the separation of extended areas of the upper epidermis specifically at the level of stratum granulosum by disruption of the desmosomes after specific cleavage of desmoglein1 (Amagai et al., 2000 ; Amagai et al., 2002 ; Piano, 2003).

MATERIALS AND METHODS

3.1. Clinical isolates & Bacterial storage

Staphylococcus aureus isolates isolated from clinical specimens in the period between the years 2000 to 2007 were kindly provided by Dr. George Araj from the American University Hospital (AUH) in Beirut-Lebanon, Dr. George Abd el-Nour from Maounat Hospital in Jbeil-Lebanon and Dr. Ziad Daoud from St George Hospital in Achrafieh-Lebanon. The reference strain ATCC 49775 carrying the Panton-Valentine Leukocidin (PVL) and γ-hemolysin (γ–*hlg*) genes was obtained from the American Type Culture Collection (ATCC). These isolates were suspended in cryobank tubes and stored at both -20°C and -80°C.

The present study was conducted on 145 isolates; AUH isolates (102 isolates) were isolated from different sites of infection, age groups and years, while the isolates from the Maounat Hospital (25 isolates) and the St George Hospital (18 isolates) were all collected from the year 2006, with ages and genders being undisclosed.

3.2. Culture conditions

The reference strain and the staphylococcal clinical isolates were inoculated onto blood and mannitol salt agar plates (Oxoid Ltd., Hampshire, United Kingdom), and incubated at 37°C for 24-48h. Mannitol fermentation was observed and recorded. *S.aureus* strains identification was performed by API Staph and Coagulase test. Colony morphology and color was also taken into consideration.

3.3. Coagulase Test

Coagulase activity was determined by the coagulase rabbit plasma test (bioMerieux, Marcy-L'Etoile, France) to differentiate *S. aureus* from the coagulase-negative staphylococci (CoNS). The test was performed by diluting the citrated plasma rabbit in freshly inoculated peptone water following the manufacturer's instructions (bioMerieux, Marcy-L'Etoile, France). The tubes were incubated at 37°C and coagulation was assessed after 24hrs.

3.4. Methicillin resistance

Methicillin resistance of all 145 samples was determined using "Slidex MRSA Detection" kit (bioMerieux, Marcy-L'Etoile, France) defined as slide latex agglutination assay that detects Penicillin-binding protein 2a, according to the protocols supplied by the manufacturer.

3.5. Staph API strips

All clinical isolates were identified to the species level using API Staph Strips (bioMérieux, Marcy-L'Etoile, France). Following the manufacturer's instructions, inoculum suspensions for the API Staph Strip (API System S.A., Montalieu-Vercieu, France) were prepared by selecting colonies from overnight growth on mannitol salt agar plates. The colonies were transferred to a specific liquid broth supplied along with the strips to produce a bacterial with a turbidity that matches that of 2 on the McFarland scale. The strips were processed after 24h of incubation at 37°C. Positive reactions were converted to a seven-digit profile number. These profiles were then compared with the index supplied by the manufacturer and with the computer database for the API Staph Strip system (APILAB PLUS v.3.3.3 1990 bioMerieux).

3.6. Hemolytic activity

Hemolysis was assessed by streaking each strain on blood base agar (Oxoid Ltd., Hampshire, United Kingdom) supplemented with 5% human defibrinated blood. Plates were incubated at 37°C for 24h. Hemolytic activity was determined based on the presence or the absence of a zone of hemolysis around bacterial colonies.

3.7. Antibiotic susceptibility testing

Antibiotic susceptibility was assessed using the disk diffusion assay. Inoculum suspensions were prepared by selecting colonies from overnight growth on mannitol salt agar plates. The colonies were transferred to a Mueller-Hinton broth (Oxoid Ltd., Hampshire, United Kingdom) to produce a suspension that matches the turbidity of a 0.5 McFarland Standard. Mueller-Hinton agar plate (Oxoid Ltd., Hampshire, United Kingdom) were inoculated with the *S. aureus* clinical isolates using a cotton swab. Each plate was stamped with 8 antibiotic diffusion disks with different concentrations: Rifampin (RD), 5µg; Clindamycin (DA), 2µg; Ciprofloxacin (CPF), 5µg; Vancomycin (VA), 30µg; Oxacillin (OX), 1µg; Oxytetracycline (OT), 30µg; Linezolid (LZD), 30µg ;and Quinupristin-Dalfopristin (QD), 15µg. Diameter of the zone of inhibition around each disk was measured following 24h of incubation at 37°C. Resistance or susceptibility profiles were determined according to Toma and Barriault (1995) and the Clinical and Laboratory Standards Institute (formerly known as NCCLS) (2000).

3.8. DNA extraction

Genomic DNA was extracted from freshly *S. aureus* inoculated mannitol salt plates using Nucleospin DNA extraction kit (Macherey-Nagel, Germany), and following the manufacturer's instructions.

3.9. PCR amplification

All PCR assays were performed on PerkinElmer GeneAmp 9700 (PerkinElmer, Wellesly, Massachusetts).

3.9.1. 16S rDNA amplification

16S rDNA amplification was used as a positive PCR control ensuring the integrity and the quality of all sample DNA used for the amplification of the PVL and γ-*hlg* genes. DNA (1.5 µl) was added to 18.5 µl of PCR mixture containing 0.4 mM dNTP, 1X PCR buffer, 2.5 mM MgCl$_2$, 0.25 µM 27F primer (5'-AGAGTTTGATCCTGGCTCA G-3'), 0.25 µM 1492R primer (5'-GGTTACCTTGTTACGACTT-3') (DeLong, 1992 ; Suzuki & Giovannoni, 1996), 1U Ampli*Taq* Gold Polymerase (Applied Biosystems, Roche) with a the following thermal cycling conditions: 2 min of initial denaturation step at 95°C, followed by 30 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min, and a final hold at 4°C. The PCR products with a positive control were electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide and visualized using a UV BioImaging system, (DigiDoc-IT system, version 2.2.0, 2003).

3.9.2. Characterization of PVL and γ-hemolysin production by *S. aureus* isolates

Primers used for the amplification of the PVL genes were: for *luk-PV-1*: 5'ATCATTAGGTAAAATGTCTGGACATGATCCA-3', and *luk-PV-2*: 5'-GCATCAASTGTATTGGATAGCAAAAGC-3', while those for the amplification of γ-*hlg* genes were: *hlg-1*, 5'-GCCAATCCGTTATTAGAAAATGC-3', and *hlg-2*, 5'-CCATAGACGTAGCAACGGAT-3'. PVL primers amplify *lukS-PV* and *lukF-PV* genes, while γ-*hlg* primers amplify *hlgC* and *hlgB* genes. Amplification was performed using the following concentrations in 50 µl reaction mix: 1.5 mM MgCl$_2$, 1X reaction buffer, 2.5 U of *Taq* polymerase,

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200 μM each deoxynucleotide triphosphate, 400 nM of each primer and 4 μl of DNA, and either the Fermentas *Taq* DNA polymerase (Fermentas, life sciences) or Ampli*Taq* Gold (Applied Biosystems, Roche). PCR thermal cycling included an initial denaturation for 2 min at 94°C and 30 cycles of a denaturation step at 94°C for 30s, an annealing step at 55°C for 30s and an extension step at 75°C for 1min. The PCR products, including a reagent control (without a template DNA) and a PCR control with *E.coli* DNA were loaded on 1.5% electrophoresis agarose gels stained by 0.5 μg/ml ethidium bromide and visualized using a UV BioImaging system, (DigiDoc-IT system, version 2.2.0, 2003).

3.10. BIOLOG gram positive identification test panel

Twenty-five clinical isolates and reference strains, representing all the API obtained numerical profiles, were identified using the BIOLOG gram positive identification test panel (BIOLOG, *Inc.*, Hayward, California). Ninety-five discrete tests were performed simultaneously in order to reveal the characteristic reaction pattern known as the "metabolic fingerprint". The metabolic fingerprint patterns were compared and identified using the MicroLog database software (release 4.01A). According to the manufacturer's instructions, a pure *S. aureus* culture was grown on tryptone soy agar medium (Oxoid Ltd., Hampshire, United Kingdom) for 16-24hrs, followed by preparing a bacterial suspension, using GP inoculating fluid (IF). IF contained 0.01% of gellan gum (Sigma), 0.04% NaCl and 0.03% a non-ionic detergent pluronic-F-68 (Sigma), supplemented with 3 drops of thioglycolate and corresponding to a total density of 20%. Bacterial suspension (150 μl) was pipetted into each well of the GP2 MicroPlate. The GP2 MicroPlate was incubated at 37°C for 16-24hrs. The GP2 MicroPlates were read and compared using the computer-controlled MicroPlate reader and the MicroLog database software (release 4.01A).

RESULTS

4.1. PVL Positive versus PVL Negative

Among the 145 *S. aureus* clinical isolates, 45 % (n=65) were PVL positive (Figure 7a) (22% (n=4) were from St George Hospital, 20% (n=5) were from Maounat Hospital and 55% (n=56) were from the AUH), while 55% (n=80) were PVL negative (Figure 7b) (Annex Tables 1a, 1b, 2a, 2b, 3a,3b).

4.2. *hlg* Positive versus *hlg* Negative

The majority of the isolates were *hlg* positive (91%) (n=132). However, the *hlg* negative isolates (9%) were from the AUH and harbored the PVL gene. (Figure 8).

4.3. PCR assays
4.3.1. 16S rDNA amplification

16S rDNA was used as a positive control to ensure the good quality of the extracted DNA, so as to be used in the PCR assays targeting the PVL and *hlg* genes. All the extracted DNA yielded a band with the expected size (1500 bp) after gel electrophoresis (Figure 9).
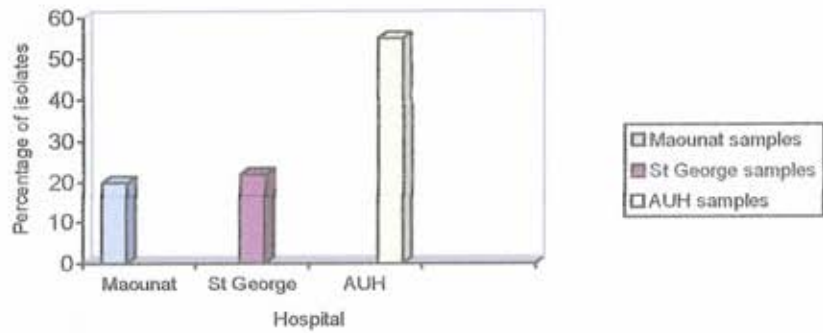
Figure 7a: Percentage of isolates collected from Maounat, St George and AUH that harbored the PVL gene.
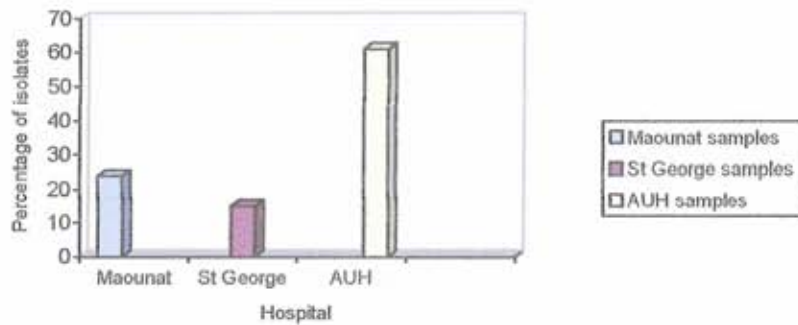
Figure 7b: Percentage of isolates collected from Maounat, St George and AUH samples and were PVL negative.
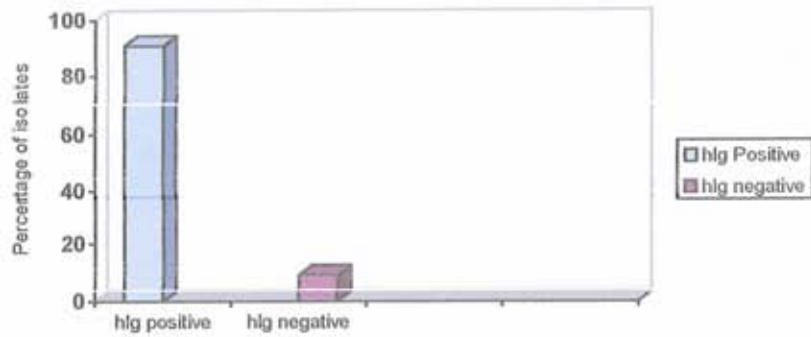
Figure 8: Percentage of isolates with *hlg* gene versus those with *hlg* negative gene.

Figure 9: 16S rDNA gene amplification.

1.5% agarose gel electrophoresis showing PCR products after 16S rDNA amplification. The first lane shows the DNA ladder, second lane: negative control containing distilled water, and the remaining lanes (3 through 10) show 16S rDNA PCR products at 1500bp.

4.3.2. PVL and γ -*hlg* amplification

PVL and γ-*hlg* genes were amplified in a duplex PCR assay using two types of *Taq* polymerases: Fermentas *Taq* DNA polymerase (recombinant) (Fermentas, life sciences) or Ampli*Taq* Gold (Applied Biosystems, Roche). ATCC 49775 was used as a reference strain, which was the positive control for both the PVL and *hlg* genes in all PCR runs. The reference strain yielded the amplification products of the expected sizes (433 bp and 937 bp) with the PVL and γ-*hlg* gene-specific primers, respectively using the Fermentas *Taq* DNA polymerase (Fermentas, life sciences) (Figure 10). However, with the Ampli*Taq* Gold polymerase (Applied Biosystems, Roche), PVL gene was detected in all isolates whether *hlg* positive or negative. Testing strains having both PVL and *hlg* genes using Ampli*Taq* Gold polymerase (Applied Biosystems, Roche), only the PVL gene was amplified while the *hlg* gene was not detected (Figure 11).

4.4. Age and PVL

Patients developing infections with PVL positive *S. aureus* were younger than those with PVL negative with a median age of 45 versus 40 years. Studying the 56 PVL positive isolates revealed that the majority of patients (62.5%) (n=35) were below the age of 55, with the highest percentage (15%) being from patients within the age group of 20-29. Moreover, 9% of the PVL positive isolates were isolated from children that were less than 5 years old, one being 1month-old while 3 others were less than 2 years old (Figure 12a); contrary to that, 58% (n=26) of PVL negative patients were older than 50 years old with 24%(n=11) belonging to the age group 60 to 69 years old and 22% (n=10) being older than 70 years old (Figure 12b).
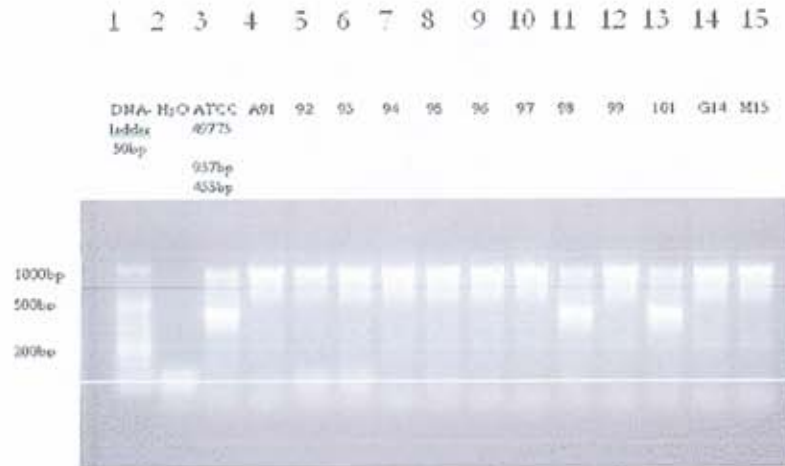
Figure 10: Duplex PCR targeting PVL and *hlg* genes using the Fermentas *Taq* DNA polymerase. Analysis on 1.5% agarose gel electrophoresis. Detection of the luk-PV genes (lanes 3-11-13) at 433bp and *hlg* (lanes 3 through 15) at 937bp. Lane 1: 50bp DNA Ladder, lane2: negative control containing distilled water, lane 3: reference strain ATCC 49775.

Figure 11: Duplex PCR targeting PVL and *hlg* genes using the Ampli*Taq* Gold polymerase. Analysis on 1.5% agarose gel electrophoresis. Detection of the luk-PV genes (lanes 3-4-7-8-9-10-11-13-14) at 433 bp and *hlg* (lanes 5-6-12) at 937bp. Lane 1 and 15: 50bp DNA Ladder, lane2: negative control containing distilled water, lane 3: reference strain ATCC 49775.
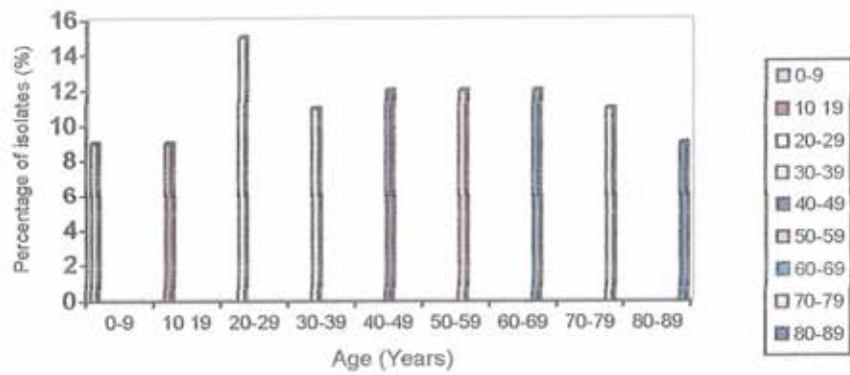
Figure 12a: The percentage of clinical isolates harboring the PVL gene falling within different age groups
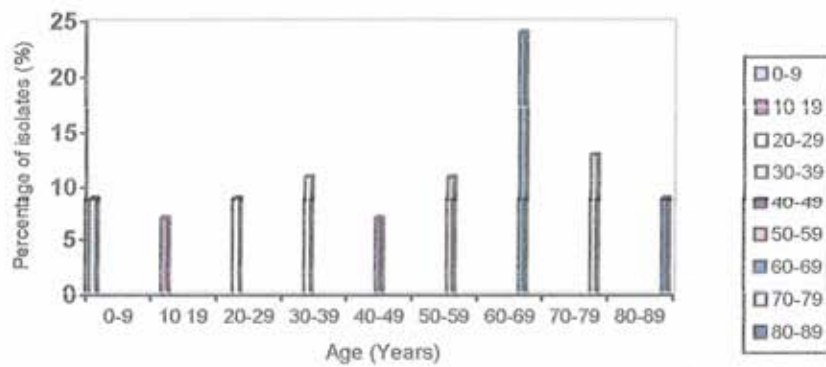
Figure 12b: The percentage of clinical isolates lacking the PVL gene falling within different age groups.

4.5. Age and *hlg*

The results obtained from patients with infections caused by *S. aureus* lacking *hlg* gene revealed that 70% (n=9) of the clinical samples with *hlg* negative were older than 30 years old, with 46% (n=6)of the population being older than 60 years old, while 15% (n=2) were younger than 1 year old (Figure 13).

4.6. Gender

Males are more prone to develop infections with *S. aureus* with 65% (n=85) of the isolates being collected from males, while only 35% (n=42) were females. Males were also more likely to develop infections with PVL positive isolates than females. Only 40% (n=23) of the infections caused by PVL positive isolates occurred in females, while 60% (n=35) in males (Figure 14a). The same applies to infections caused by *hlg* negative isolates, where 31% (n=4) were isolated from females and 69% (n=9) from males (Figure 14b).

4.7. Site of infection

Wounds were the predominant site of staphylococcal infections, with 42% (n=61) of strains being recovered from wounds (Figures 15a, 15b). Added to that, the majority of samples recovered from wounds (61%) (n=37) harbored the PVL gene as opposed to 39% (n=24) of PVL negative isolates. On the other hand, 13% (n=19) of the *S. aureus* isolates were isolated from pus, with 42% (n=8) being PVL positive and 58% (n=11) PVL negative (Figures 15a, 15b). *S. aureus* causing superficial skin abscesses represented 6% (n=10) of all infections caused by this organism and 80% (n=8) of those were found to be PVL positive (Figure 15a). *S. aureus* from

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sputum formed 4% (n=6) of the studied population, within which only 16.7% (n=1) were PVL positive. PVL negative isolates were also isolated from blood (8%), stool (5.3%), nasal (1.3%), urine (5.3%), brain (1.3%), joint fluid (1.3%) throat swab (4%), ear swab (1.3%), neck mucus (1.3%), breast (1.3%), DTA (6.7%) and tissue (1.3%), thus constituting 37.3% (n=28) of all PVL negative *S.aureus* isolates (Figure 15b). It is noteworthy that two distinct *S.aureus* species (1.4%) were isolated from catheters, with one carrying the PVL gene and the other being PVL negative. Other sites of infection from which PVL positive isolates were isolated included DTA (7.2%), blood (4.4%), gall bladder (1.4%), cyst (1.4%), nasal (1.4%), fluid (1.4%), eye (1.4%), BRW (1.4%) and thumb (1.4%) (Figure 15a). However, *hlg* negative isolates were primarily isolated from wounds (46% ; n=6), and skin superficial abscesses (15% ; n=2).

4.8. Year of infection

PVL negative isolates were prevalent among the isolates recovered in the year 2006, representing 70.7% (n=53) of tested isolates (Figure 16a). During the years 2000 till 2002, 76% (n=38) of the 53 isolates were PVL positive (Figure 16a) and 61.5% (n=8) were *hlg* negative. However, during the years 2005 to 2007, 32.25% (n=30) of the 93 clinical isolates harbored the PVL gene, while 67.75% (n=63) lacked the PVL gene (Figure 16b) and 38.5% (n=5) were *hlg* negative.
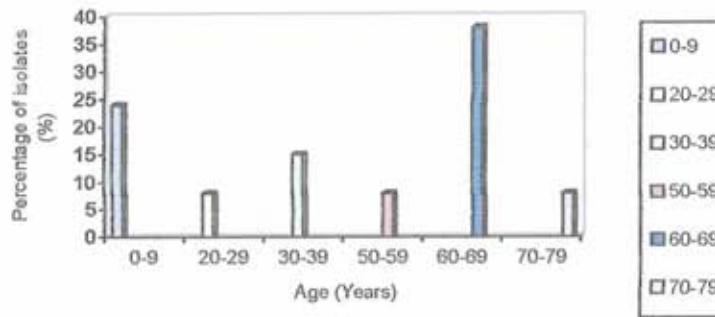
Figure 13: The percentage of clinical isolates with *hlg* negative gene falling within different age groups
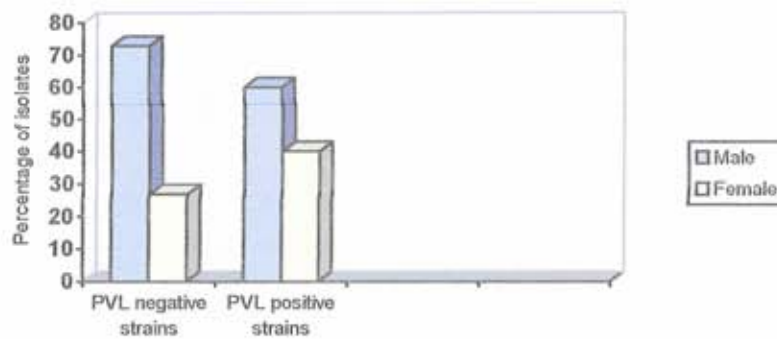
Figure 14a: Percentage of isolates isolated from male and female patients that were either PVL positive or negative.
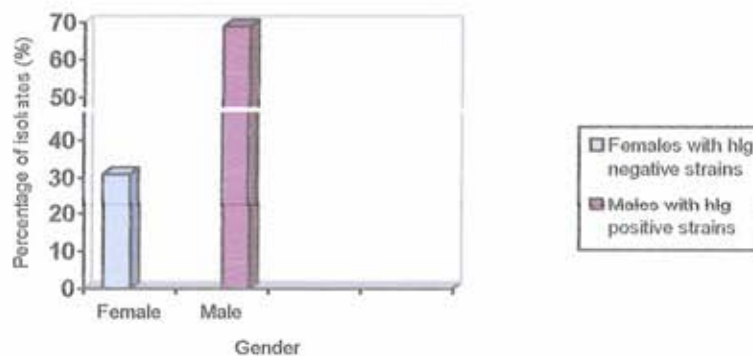
Table 14b: Percentage of isolates with *hlg* negative gene isolated from males and females patients.
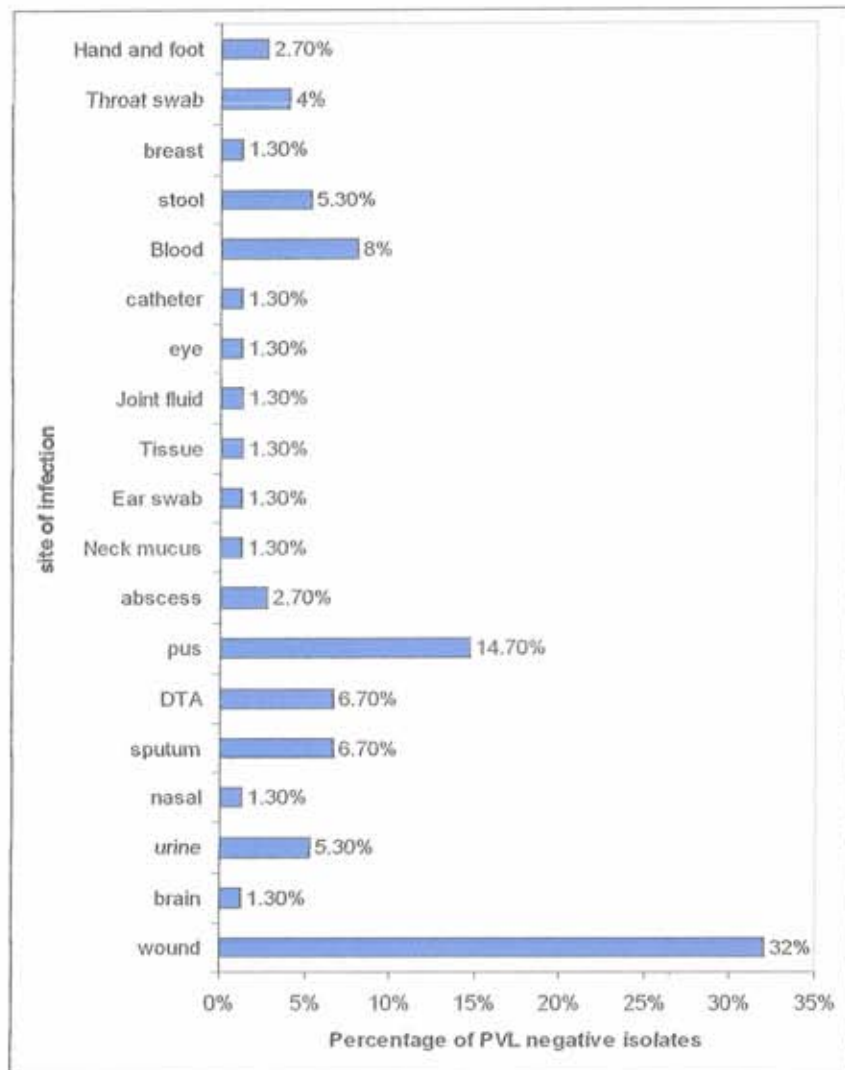
Figure 15a: Percentage of PVL negative isolates isolated from various sites of infection.
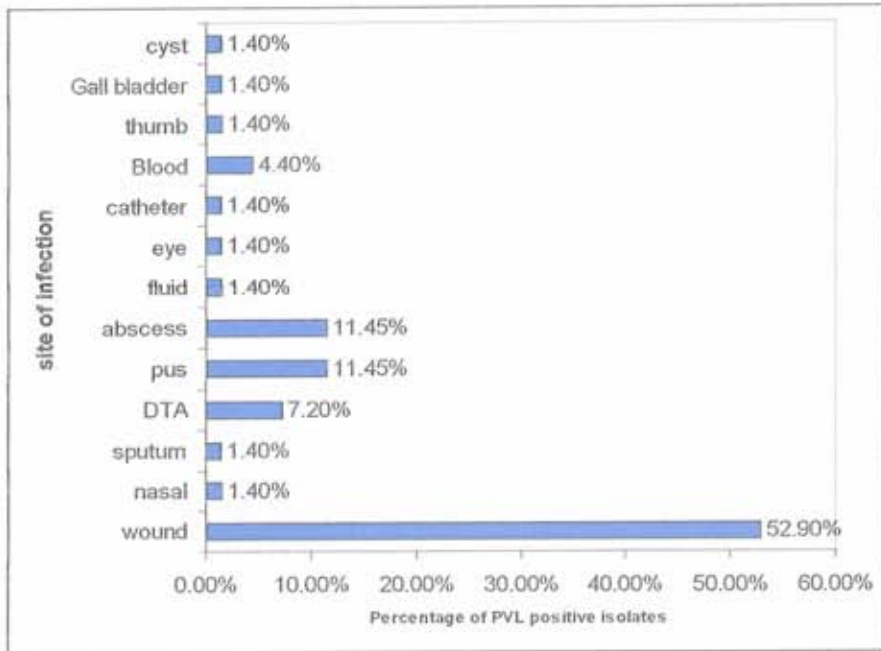
Figure 15b: Percentage of PVL positive isolates isolated from various sites of infection.
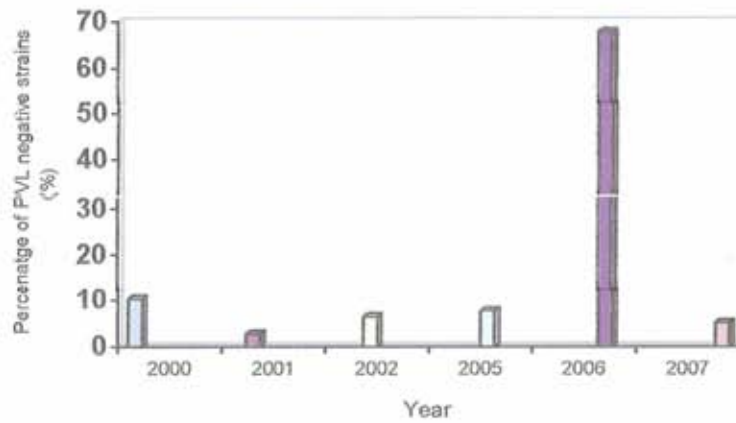
Figure 16a: Percentage of PVL negative isolates isolated from different years
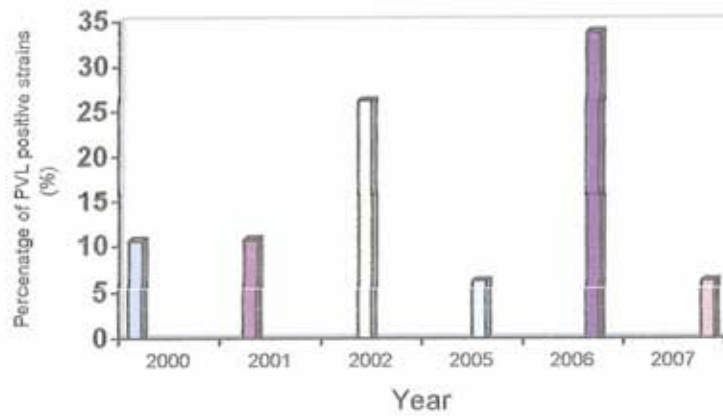
Figure 16b: Percentages of isolates harboring PVL gene isolated in different years

4.9. PVL and MRSA/MSSA

Out of the 80 PVL negative isolates, 62.5% (n=50) were MSSA, while 81.5% (n=53) of the *S. aureus* isolates that harbored the PVL gene were MRSA (Figure 17).

4.10. Colony Morphology

Colonies, grown on mannitol salt agar, were distinguished according to their color and morphology. Two different types of colonies were detected: the first was characterized by being small and white, while the second variant was large and orange pigmented. Orange colonies represented 69% (n=100) of all isolated clinical samples and white colonies constituted 31% (n=45) of the samples (Figures 18a, 18b). A correlation was established between those morphological characteristics, the presence or absence of PVL and methicillin resistance or susceptibility. Out of the 80 PVL negative isolates, 62.5% (n=50) were MSSA and 37.7% (n=30) were MRSA. Of the 50 MSSA and PVL negative isolates, 48% (n=24) were white colonies and 52% (n=26) were orange (Figure 19a). Within 30 MRSA and PVL negative isolates, 40% (n=12) were white and the remaining 60% (n=18) were orange pigmented colonies (Figure 18b). On the other hand, of the 65 PVL positive isolates, 16.9% (n=11) were MSSA while 83.1% (n=54) were MRSA. Out of the 11 MSSA and PVL positive isolates, only 27.3% (n=3) were orange colonies, while 72.7% (n=8) were white. Moreover, within the 54 MRSA and PVL positive isolates, only 9.3% (n=4) were white while the majority 90.7% (n=50) appeared as orange pigmented colonies (Figure 19b).
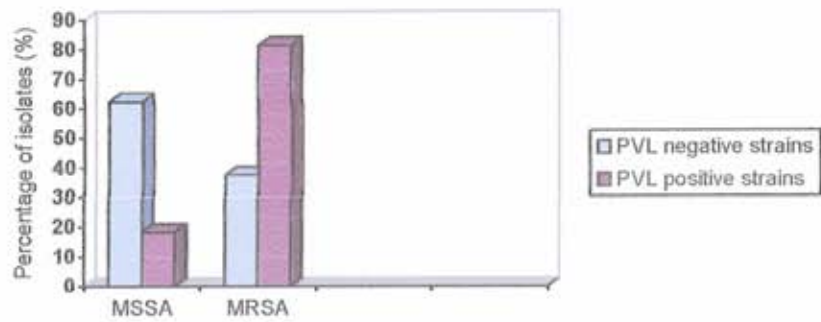
Figure 17: Relation between PVL positive and/or negative *S. aureus* isolates versus MSSA and/or MRSA

Figure 18a: Non-pigmented *S. aureus* isolates producing tiny and white colonies on mannitol salt agar.
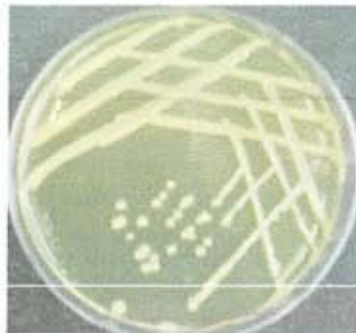
Figure 18b: Carotenoid-pigmented *S. aureus* isolates producing large and orange colonies on mannitol salt agar.
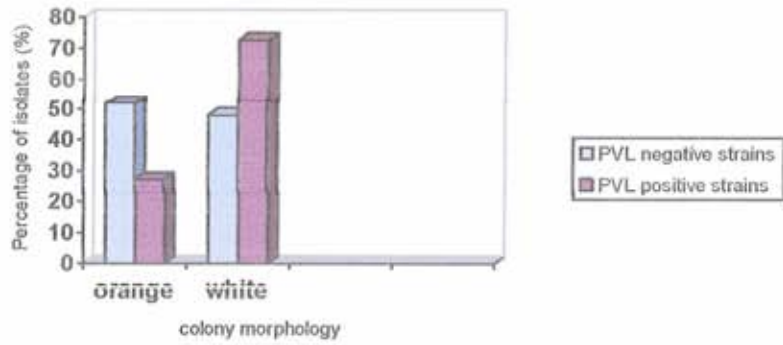
Figure 19a: Relation between MSSA isolates, colony morphology and the occurrence of PVL gene.
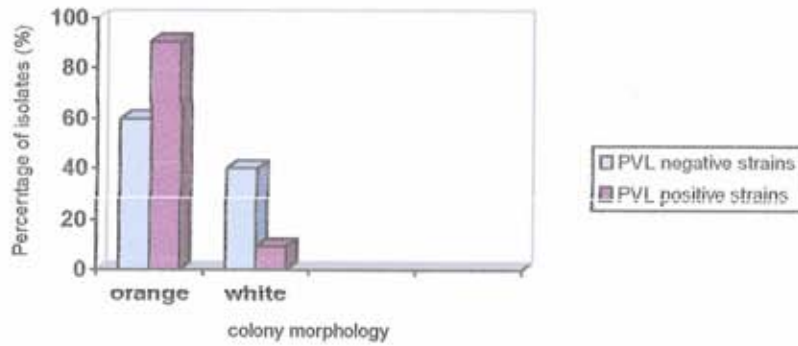
Figure 19b: Relation between MRSA isolates, colony morphology and the occurrence of PVL gene.

4.11. Coagulase Test

All *S. aureus* clinical isolates (98.62% ; n=143) were coagulase positive except one isolate taken from St George Hospital and another from Maounat Hospital, which were identified as *S.saprophyticus* and *S.warneri*, respectively. Thus 98.62% (n=143) coagulated using the Rabbit plasma test.

4.12. Latex agglutination test

Out of the 145 isolates, 59% (n=85) were MRSA.

4.13. Hemolysis

The majority (75.2% ; n=109) of the S. *aureus* clinical isolates were hemolytic. Hemolysis was also correlated with other morphological and genetic characteristics, where 50% (n=72) of the orange pigmented colonies and 97.24% (n=106) of the *hlg* positive isolates were hemolytic. On the other hand, 55% (n=60) of the PVL negative isolates were hemolytic on blood agar. However, out of the 36 samples that were non-hemolytic, 50% (n=18) were PVL positive, 2.78% (n=1) were *hlg* negative and 25% (n=9) were non-pigmented colonies.

4.14. API system identification

Using the computer database for the API Staph Strip system, 6 profiles of *S. aureus* were detected, while one isolate from St George Hospital was identified as *Staphylococcus saprophyticus* and another isolate from Maounat Hospital as *Staphylococcus warneri*. *S. aureus* numerical profiles found in the tested population included: Numerical profile I (44.8%, n=65), II (50.3%, n=73), III (0.7%, n=1) IV (0.7%, n=1), V (1.4%, n=2), VI (1.4%, n=2) and VII (1.4%, n=2) (Figure 20a) (Annex Table 4). Lactose utilization served to differentiate between the major two numerical profiles I and with 50.3% of the clinical isolates being able to ferment lactose. Another

correlation existed between these various numerical profiles and the occurrence of PVL gene, where 85% (n=68) out of the 80 PVL negative isolates had the numerical profile II, 5% (n=4) I, 2.5% (n=2) III, 2.5% (n=2) VI, 0.7% (n=1) IV and 2.5% (n=2) VII (Figure 20b). As for the 65 PVL positive isolates, 70.77% (n=46) had the numerical profile I and 27.7% (n=18) had the numerical profile II (Figure 20c).

4.15. Antibiotic susceptibility testing

Eight antibiotics were used to detect susceptibility and resistance of the clinical isolates undertaken in this study. Antibiotics tested were: Rifampicin (RD) 5µg, Clindamycin (DA) 2µg, Ciprofloxacin (CPF) 5µg, Vancomycin (VA) 30µg, Oxacillin (OX) 1µg, Oxytetracycline (OT) 30µg, Linezolid (LZD) 30µg, and Quinupristin-Dalfopristin (QD) 15µg (Figures 21a, 21b, 21c, 21d, 21e).

According to Figure 22a, 6.2% (n=9) were equally resistant to rifampicin and quinupristin-dalfopristin, 7.6% (n=11) to clindamycin and 13.1% (n=19) to ciprofloxacin. Low resistance percentages were detected to vancomycin (2.7% ; n=4) and linezolid (2.1% ; n=3). However, resistance to oxacillin and oxytetracycline constituted the highest percentages 31.03% (n=45) and 46.2% (n=67), respectively.

PVL negative versus positive isolates exhibited a distinct pattern of antimicrobial susceptibility and resistance. Among the PVL negative isolates, resistance was seen to all 8 antibiotics tested, while PVL positive were all susceptible to vancomycin and linezolid. Among the 145 clinical isolates, 9 samples (6.2%) were resistant to rifampicin; out of which 7 (8.75%) were PVL negative and 2 (3%) were PVL positive (Figures 22b, 22c). Eleven isolates (7.6%) revealed resistance to clindamycin, within which 10 isolates (12.5%) were PVL negative and only one (1.5%) was PVL positive (Figure 23a, 23b). The majority of tested isolates were susceptible to vancomycin (97.2%; n=141) which inhibits cell wall synthesis, and linezolid (97.9%; n=142) which affects protein synthesis.

numerical profile II

numerical profile I

numerical profile V
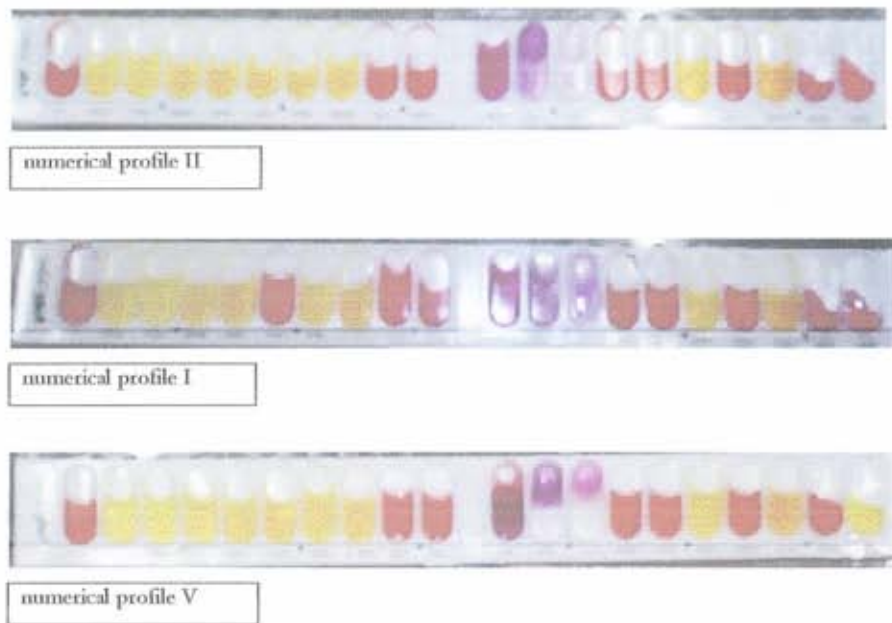
Figure 20a. API strips showing different profiles
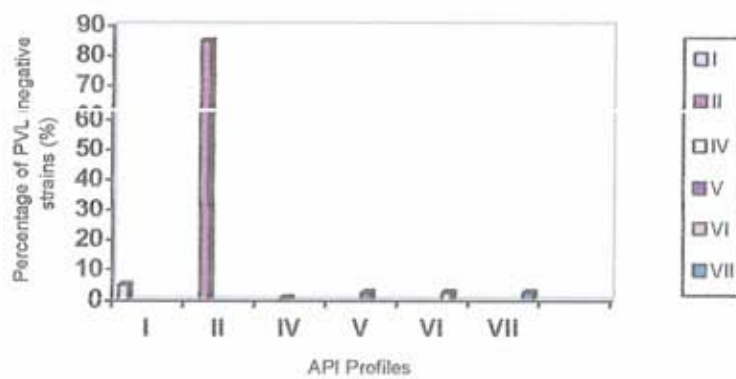
Figure 20b: The percentage of PVL negative isolates showing various API profiles.
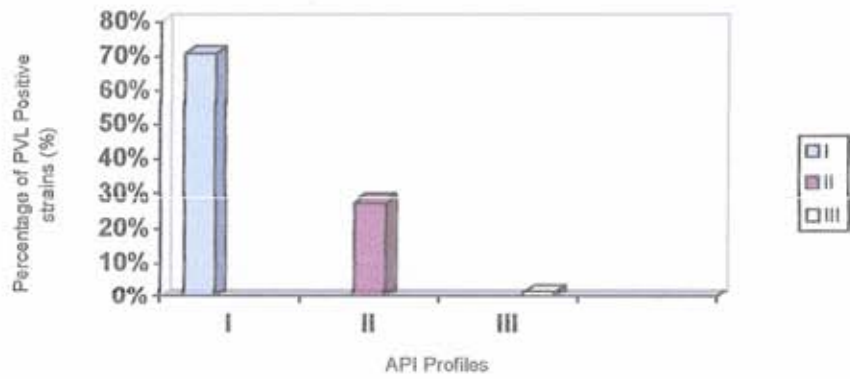
Figure 20c: The percentage of PVL positive isolates showing various API profiles.
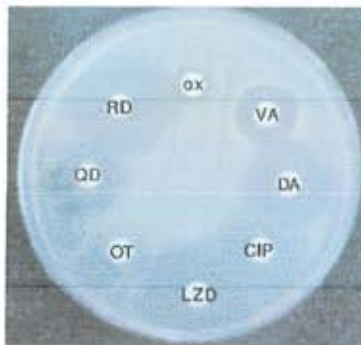
Figure 21a: Antibiotic disk-diffusion test applied to *S.aureus* isolate showing resistance to oxacillin. The antibiotics used were: oxacillin (OX) 1 μg, vancomyin (VA) 30 μg, clindamycin (DA) 2 μg, ciprofloxacin (CIP) 5 μg, linezolid (LZD) 30 μg, oxytetracycline (OT) 30 μg, rifampicin (RD)5 μg and quinupristin-dalfopristin (QD) 15 μg.
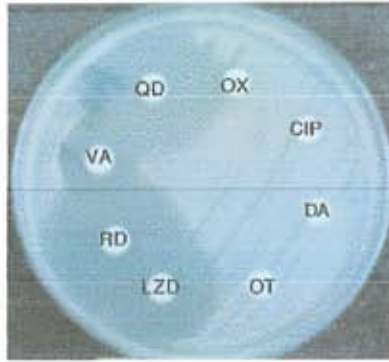
Figure 21b: Antibiotic disk-diffusion applied to *S.aureus* isolate showing resistance to oxacillin, ciprofloxacin, clindamycin, and oxytetracycline.
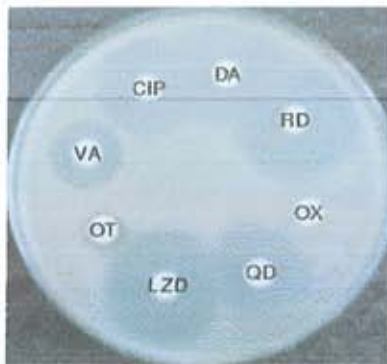
Figure 21c: Antibiotic disk-diffusion applied to *S.aureus* isolate showing resistance to oxacillin.
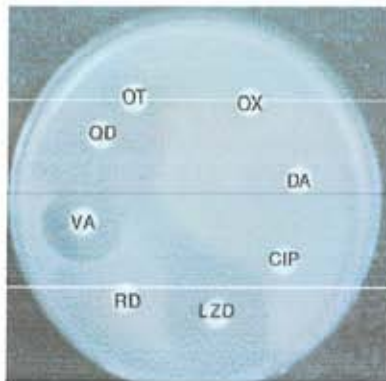
Figure 21d: Antibiotic disk-diffusion applied to *S.aureus* isolate showing resistance to oxacillin, ciprofloxacin, clindamycin and rifampicin.
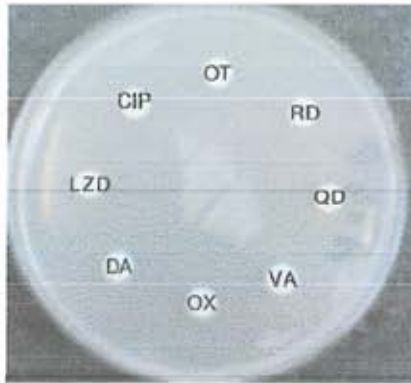
Figure 21e: Antibiotic susceptibility of *S.aureus* isolate using the disk-diffusion assay.

The four vancomycin resistant isolates and the three linezolid resistant ones were all PVL negative (Figure 23a, 23b). Oxacillin and oxytetracycline resistant isolates constituted a large proportion of the studied *S.aureus* strains, with 31.03% (n=45) being oxacillin resistant, and 60.69% (n=66) being oxytetracycline resistant. Added to that, the percentage of PVL positive isolates resistant to these antibiotics was significantly higher than that of PVL negative isolates (for OX 46.2% to 18.75%; for OT 69.2% to 27.5%) (Figures 23a, 23b). In the case of ciprofloxacin, 13.1% (n=19) of the isolates were resistant; out of which 7.7% (n=5) harbored the PVL gene. Moreover, for quinupristin-dalfopristin, known to interfere with protein synthesis, 93.79% (n=136) of all 145 isolates were susceptible; 4.6% (n=3) of the PVL positive isolates and 7.5% (n=6) of the PVL negative isolates were resistant to quinupristin-dalfopristin (Figures 23a, 23b).

On the other hand, multiple antibiotic resistance was mainly detected in PVL negative isolates. As such, a single isolate (1.25%) was found resistant to seven antibiotics, and susceptible only to linezolid. However, 5% (n=4) of the isolates were resistant to five antibiotics, 7.5% (n=6) to four antibiotics and 6.25% (n=5) to three antibiotics (Figure 22b, 22c). PVL positive isolates were not resistant to more than four drugs (3.1% , n=2), 1.5% (n=1) were resistant to three drugs, and the majority (76.92% , n=50) were resistant to one (38.5% , n=25) or two drugs (38.5% , n=25). In contrast, a much lower percentage (15%, n=12) of the PVL negative isolates were resistant to one (10%, n=8) or two (5%, n=4) drugs. It is interesting to note that 45.5% (n=66) of all tested strains were susceptible to all eight used antibiotics, with 35.86% (n=52) being PVL negative (including the *S.saprophyticus* and the *S.warneri*) and 9.65% (n=14) were PVL positive. Finally, none of the 145 *S.aureus* clinical isolates was resistant to all tested antimicrobial agents (Figures 22b, 22c).
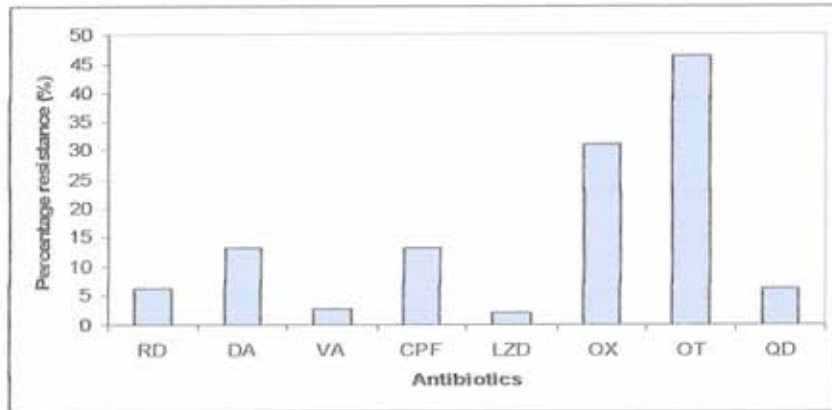
Figure 22a: Percentage resistance of the *S. aureus* clinical isolates to different antimicrobial agents.
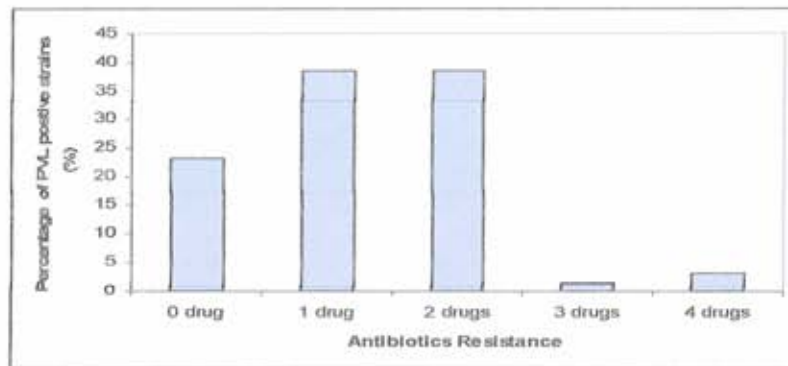
Figure 22b: Percentage resistance of the PVL positive *S. aureus* clinical isolates to none, one or more drugs of the tested antibiotics
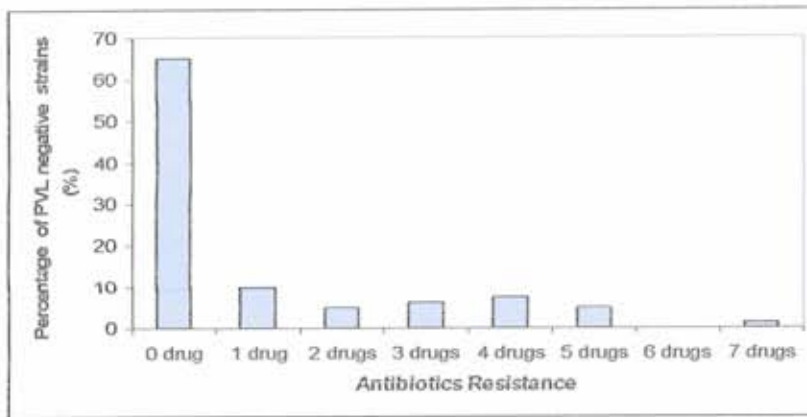
Figure 22c: Percentage resistance of the PVL negative *S. aureus* clinical isolates to none, one or more drugs of the tested antibiotics.
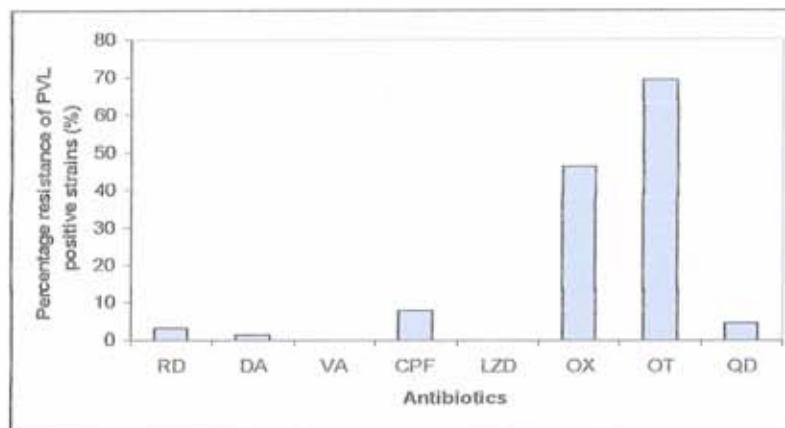
Figure 23a: Percentage resistance of the PVL positive *S. aureus* clinical isolates to various antibiotics.
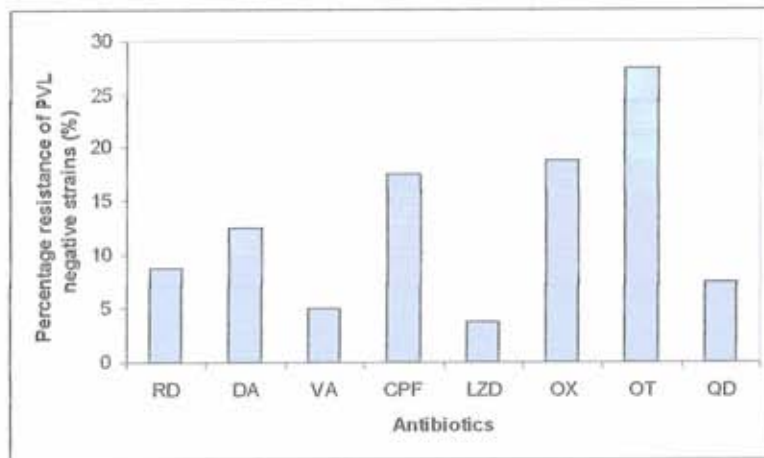
Figure 23b: Percentage resistance of the PVL negative *S. aureus* clinical isolates to various antibiotics.

4.16. BIOLOG Identification of *S. aureus*

Color development in every well of the GP MicroPlate reflected the ability of the bacterial strain to utilize a specific carbon source. After identifying 143 clinical isolates as *S.aureus* with API Staph Strips, 25 were chosen to represent the different recovered API profiles. According to the results obtained, 40% (n=10) of the isolates were successfully identified at the species level, 36% (n=9) were identified at the genus level, while 24% (n=6) were not identified. However, repeating the test for those isolates giving no ID led to successful identification and gave not only the ID at the genus level but also at the species level. Results obtained after repeating the test were more accurate than the initial results. Additionally, and in order to test the reproducibility of the BIOLOG system, each isolate was tested twice, and duplicate MicroPlates were used to determine the ID of each strain. Results from the duplicates in the majority of the cases didn't match.

4.16.1. Comparison between the identification results using the API Staph strips and BIOLOG MicroPlates

The major difference between the two dominant API profiles assessed among the 145 clinical isolates was a single lactose test, as stated previously. Therefore, lactose utilization was followed up on the two used systems API Staph and BIOLOG. BIOLOG results obtained didn't always match with that of the API Staph strips. The lactose test was negative in all of the 25 BIOLOG tested strains, while the D-glucose and D-Fructose tests were positive in all BIOLOG and API Staph tested strains. D-Mannose test was positive in all API Staph tested isolates and in the correctly identified BIOLOG isolates at the species level. Those that were labelled as no ID or were only identified at the genus level had a negative D-Mannose test. In contrast, the Maltose test was 100% positive in all isolates tested using the API Staph and of all isolates identified as

S.aureus using the BIOLOG system. However, this test was negative in 77.78% (n=7) of those identified at the genus level and in 16.67% (n=1) of the isolates that were not successfully identified using the BIOLOG.

DISCUSSION

Staphylococcus aureus, is a major pathogen causing a wide spectrum of clinical manifestations and is associated with both nosocomial and community-acquired infections (Francis et al., 2004). Over the past few years, the dramatic increase in the occurrence of community-acquired methicillin resistant *S. aureus* (CA-MRSA) has triggered an intense research for the underlying factors (Voyich et al., 2006). CA-MRSA diseases were found to be more severe than those caused by Hospital-acquired MRSA (HA-MRSA) due to their association with the occurrence of a particular gene known as Panton-Valentine Leukocidin (PVL) (Kaneko & Kamio, 2004 ; Voyich et al., 2006).

PVL, a pore-forming cytotoxin that targets human and rabbit mononuclear and polymorphonuclear (PMN) cells without any hemolytic activity, has dermonecrotic and cytolytic properties (Prevost et al., 2001 ; Morinaga et al., 2003 ; Viero et al., 2006). The results of this study showed that among 145 *S. aureus* clinical isolates, 45% harbored the PVL gene. Other studies, however such as those conducted by Ramdani-Bouguessa et al. (2006) in Algeria, revealed that PVL gene was the most frequently detected toxin gene (72% of *S. aureus* isolates), whereas 35% of clinical isolates in Capes Verde Islands (Aires-de-Sousa et al., 2006), and 37% in Lyon France (Lina et al., 1999) were tested PVL positive.

On the other hand, Vandenesh et al. (2003) showed that 67% of CA-MRSA isolated from France and Switzerland, 29% of those from the USA, 13% from Oceania Southwest Pacific and none from Australia Queensland harbored the PVL gene. Furthermore, Holmes et al. (2005) reported that only a small proportion (1.6%) of 9000 *S. aureus* clinical isolates collected throughout England and Wales were PVL positive, while Prevost et al. (1995) found that the PVL gene was detected in 2% of *S. aureus* isolates in a general hospital in France and in less than 5% of all *S. aureus* isolates in western Europe. This discrepancy in reports describing PVL as an infrequent toxin in *S. aureus* was probably due to the differences in geographic areas and patients selection (random patients versus patients with community acquired soft tissue abscesses) (Issartel et al., 2005).

The majority of *S.aureus* isolates (99%) were found to be *hlg* positive (Lina et al., 1999). In this study, 91% of the isolates were *hlg* positive, with 9% of the *hlg*-negative samples harboring the PVL gene. *hlg* and PVL, form a bi-component synergohymenotropic pore-forming toxins acting in synergy but being separately secreted (Prevost et al., 1995). Hence, PVL positive and *hlg* negative isolates are capable of acting on PMN cells but unable to lyse erythrocytes.

PVL has been identified as a stable marker of CA-MRSA isolates worldwide (Holmes et al., 2005), with our results being consistent with this finding where the majority (81.5%) of the PVL positive tested *S. aureus* isolates were MRSA. Bocchini et al. (2006) also reported that 94.9% of the *S. aureus* harboring the PVL gene were MRSA, while Kuehnert et al. (2006) found that a much lower percentage of the MRSA isolates harbored the PVL gene being (8% in the USA).

McClure et al. (2006) suggested that newly emerging CA-MRSA carried the PVL virulence gene and possessed a small mobile genetic element the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV or V, which harbors the methicillin resistance gene (*mecA*) and was more easily transferred to other *S. aureus* isolates than the larger SCC*mec* types I, II and III that prevail in HA-MRSA isolates. According to Jabbour (2007) working on the same isolates undertaken in this current study, 89.2% of the PVL positive MRSA belonged to SCC*mec* type IV and the remaining were SCC*mec* type V. This combination of PVL with the *mecA* gene appears to have created a superadapted "ecologically fitted" *S. aureus* strain that was spreading in the community due to their short doubling time (Dufour et al., 2002 ; Maree et al., 2007). However, Vonberg et al. (2006) recommended a number of approaches that could limit nosocomial MRSA spread such as educating hospital staff, sufficient alcohol-based hand disinfection, and early placement of MRSA-positive patients in isolation. Nevertheless, Voyich et al. (2006) pointed out that the multiple factors in the overall genetic makeup of each isolate, rather than any single determinant like PVL, promotes CA-MRSA infections and that although PVL was a useful marker for the potential of the isolates to cause severe infections, its clinical significance has not yet been determined and it could not be considered as a major determinant of disease caused by CA-MRSA strains. In contrast, Etienne (2005) considered that in the future, screening for the PVL virulence gene might become a routine laboratory procedure, while Vandenesh et al. (2003) asserted that PVL gene was a virulence determinant.

PVL has a dermonecrotic effect on both intact and damaged skin, damaging host cell membranes by the synergistic action of two classes of secretory proteins designated S and F (Bocchini et al., 2006). Lina et al. (1999) established that PVL is associated with deep seated skin infections,

furunculosis (93% of cases) and necrotizing pneumonia (85% of cases). This study revealed that 52.9% of the PVL positive isolates were collected from skin wounds, while Denis et al. (2005) found that 2.4% of PVL positive isolates were isolated from wounds. Lina et al. (1999) showed that 11.45% of the PVL positive isolates were collected from abscesses and pus, while Johnsson et al. (2004) indicated that 6.25% of PVL positive isolates were from abscesses. On the other hand, Moroney et al. (2006) showed that PVL negative isolates were predominantly recovered from skin, wound and abscesses. However, Issartel et al. (2005) revealed that there was no difference in the prevalence of PVL between *S. aureus* isolates recovered from primary and secondary infections, with PVL negative patients being more likely to have *S. aureus* blood infection. Our results were in contrast with Bocchini et al. (2006) findings, who reported that the percentage of PVL positive isolates from blood was significantly higher than that of PVL negative strains (67.2% versus 19.2%, respectively). However, Johnsson et al. (2004) pointed out that PVL could not be considered as an important virulence factor in invasive bloodstream infections. Intravascular catheters, which are crucial for the treatment of chronically ill subjects, could complicate infections by increasing duration of hospitalization, morbidity and additional medical costs (Cicalini et al., 2004). Preventive strategies, such as impregnating catheters with antimicrobial agents, were implemented in order to reduce previously stated risks (Cicalini et al., 2004), which possibly explained the low prevalence (1.4%) of *S. aureus* strains colonizing catheters in this study.

Age and gender differences were another important aspect detected in this study between PVL negative and positive isolates, with the latter being predominant (62.5%) in patients below 55 years old, as opposed to PVL negative isolates that occur mostly (58%) in subjects above 50 years old. Denis et al. (2005) stated that the median age of patients with *S. aureus*

infections harboring PVL gene was 24 years, while Wannet et al. (2005) reported that the median age in the Netherlands was 31 in the year 2000 and 25 in 2002, and that it was 48 years in California, USA, according to Maree et al. (2007) and 25 years in Sweden (Johnsson et al., 2004). Lopez-Aguilar et al. (2007), and in contrast with the results of this study, found that the median age of patients developing infections with PVL positive isolates was 67 years, while that of patients with PVL negative isolates was 76 years in Spain. However, Kilic et al. (2006) asserted that the PVL recovery was statistically higher in children (98.1%) than in adults (89.1%), and Etienne et al. (2005) described that no specific age group or population could be attributed to the occurrence of PVL, although there was a slight tendency towards younger age groups. Finally, Kilic et al. (2006) revealed that infections with PVL strains were statistically higher in children than in adults.

Gender was another important factor and according to our results, males (65%) were more prone to establish *S. aureus* infections with isolates harboring the PVL gene. In harmony with our findings, Johnsson et al. (2004), Kuehnert et al. (2005) and Lopez-Aguilar et al. (2007) found out that males (60%) were at higher risk to develop infections caused by PVL positive *S. aureus* isolates, while Denis et al. (2005) reported that female patients with PVL positive *S. aureus* isolates constituted 56% of PVL positive *S. aureus* infections.

Furthermore, in this study, the percentage of the occurrence of PVL among *S. aureus* isolates has notably decreased from the year 2000-2002 compared to 2005-2007 (from 76% to 32%, respectively). Maree et al. (2007), in contrast, revealed that the percentage of MRSA belonging to SCC*mec* type IV that were characterized by having the PVL gene followed an ascending trend from the year 1999 to 2004, peaking in the year 2003.

Xue Ma et al. (2006), on the other hand, noticed that the percentage of PVL gene occurrence has significantly decreased from the years 1979 and 1985 to 1992 and 1999 (45.3% to none). In addition, in Algeria, the percentage of PVL gene has increased up to 66% during the period between 2001-2004 (Bocchini et al., 2006) and up to 72% in the period between 2002-2003 (Ramdani-Bouguessa et al., 2006).

Drug resistance patterns were different among PVL positive and PVL negative *S. aureus* isolates. Among the 145 *S. aureus* isolates tested in the present study, oxacillin and oxytetracycline were the least effective drugs. PVL positive strains exhibited a higher resistance to oxacillin and oxytetracycline (46.2% and 69.2% respectively) than PVL negative *S. aureus*, while AnDiep et al. (2004) found that 27% of *S.aureus* isolates resistant to tetracycline were PVL positive. Oxacillin resistance was estimated to be 31.03%. Shittu & Lin (2006) revealed that oxacillin resistance was 26.1% in South Africa, while it was 47% in England and Wales (Holmes et al., 2005). We confirmed through the latex agglutination test that the oxacillin resistant isolates were MRSA, and Jabbour (2007) previously reported these clinical isolates harbor the *mecA* gene coding for methicillin resistance via penicillin binding protein 2a.

Oxytetracycline was another important antibacterial agent used in this study. Oxyetracyclines exhert antibacterial activity by interacting with the bacterial 30S ribosomal subunit, thus inhibiting protein synthesis (Denis et al., 2006). Oxytetracycline resistance was estimated in this study to be 46%. It was however, 34% in Germany (Strommenger et al., 2003), 92% in Michigan (LaPlante et al., 2007), and none in Australia where all MRSA were susceptible to tetracyclines (Collignon et al., 1998). Added to that like the Lebanese clinical isolates, European isolates appeared to be more resistant to tetracyclines than the Oceanian and US isolates

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(Vandenesh et al., 2003). However, vancomycin and linezolid were the most effective antibiotics tested in our study, with less than 3% resistance being mainly detected within PVL negative isolates. Tiwari & Sen (2006) reported that the resistance to vancomycin was 0.7% in the northern part of India, whereas Francois et al. (2004) did not detect any vancomycin resistant strains in Switzerland. This was also the case in Japan (Yamamoto et al., 2006 ; Piao et al., 2005), Belgium (Denis et al., 2006), Arkansas, USA (Ruhe et al., 2007), Saudi Arabia (Baddour et al., 2006), Canada (Mulvey et al. 2005), England and Wales (Holmes et al., 2005). Thus, vancomycin remains the drug of choice of staphylococcal infections (Srinivasan et al., 2002 ; Stryjewski et al., 2006). Vancomycin resistance developed as the result of a probable conjugal transfer of the *vanA* operon from a vancomycin-resistant *Enterococcus faecalis* to *S.aureus* (Sinsimer et al., 2005). Additionally, cell wall thickening by the increased production of peptidoglycan, which traps vancomycin molecules before getting to the cytoplasmic membrane where peptidoglycan synthesis occurs, could be one of the mechanism involved in resistance development (Lowy, 2003).

Only 2% of the tested *S.aureus* isolates in this study were linezolid resistant. Similarly, Baddour et al. (2006) pointed out that linezolid effectively inhibited the growth of 95.9% of *S.aureus* strains in Saudi Arabia, which was the case in Canada (Mulvey et al., 2005), Poland (Matynia et al., 2005) and UK (Wilson et al., 2006), where no resistance to Linezolid was detected. Moreover, and in a recent study, linezolid was considered an effective bacteriostatic agent that has the potential to reduce hospital stay length and cost (Bassetti et al., 2005 ; Lowy, 2003). Susceptibility to rifampicin and quinupristin-dalfopristin was another important finding in this study as both exhibited low resistance profiles (6%). In England and Wales, less than 3% of *S.aureus* isolates were

susceptible to quinupristin-dalfopristin (Holmes et al., 2005). Similar results were reported by Baddour et al. (2006) and Denis et al. (2006), where all isolates recovered from Saudi Arabia (Baddour et al., 2006) and from Belgium (Denis et al., 2006) were susceptible to quinupristin-dalfopristin, which is considered as a new antimicrobial being active against drug-resistant staphylococci (Lowy, 2003). Rifampicin susceptibility was also confirmed in Belgium (97.1%) (Denis et al., 2006), in Canada (100%) (Mulvey et al., 2005) and in Lyon France (97.8%) (Ramdani-Bouguessa et al., 2006). *S.aureus* strains recovered in this study were found to be susceptible to clindamycin (7.6%) and ciprofloxacin (13.1%). Resistance to ciprofloxacin was less than 3% in the United Kingdom (Holmes et al., 2005), not detected in Canada (Mulvey et al., 2005) or Belgium (Denis et al., 2006), while it was 24% in Detroit (LaPlante et al., 2007) and 27% in Arkansas (Ruhe et al., 2007). Susceptility of the PVL positive strains to ciprofloxacin in the present study was not the same as that detected in San Francisco (86.9% versus 70%) (AnDiep et al., 2004) and in Japan (100%) (Yamamoto et al., 2006).

On the other hand, 7.6% of our tested strains were clindamycin resistant compared to 38.6% in Belgium (Denis et al., 2006) and 2% in Arkansas (Ruhe et al., 2007). Yamamoto et al. (2006) reported that 27.3% of the PVL positive strains were resistant to clindamycin, while it was only 1.5% in this study. Takizawa et al. (2005), reported that 60% of the PVL negative *S.aureus* isolates were clindamycin resistant in Japan, while according to the results of this study, the percentage of PVL negative isolates resistant to clindamycin didn't exceed 12.5%. The emergence of antibiotic resistance in developing countries seems to be very much related to the irrational antibiotic usage due to its easy availability at the drug store without prescription, high rate of self-medication, injudicious use in hospitals and uncontrolled use in agriculture, animal husbandry

and fisheries (Tiwari & Sen, 2006). Furthermore, multiple drug resistance in PVL negative isolates was detected in this study, which was in harmony with Kilic et al. (2006) findings. However, 82.8% of the PVL negative isolates were CA-MRSA (Jabbour, 2007), which was in accord with Boyle-Vavra et al. (2005), who reported that around 61% of the CA-MRSA were resistant to at least four classes of the non β-lactam antibiotics. Gandara et al. (2006), however, found that CA-MRSA possessed multiple toxin genes that were not present in the genomes of HA-MRSA, which further proved that CA-MRSA were generated independently of HA-MRSA by inserting SCC*mec* into the genomes of virulent *S.aureus* isolates, thus resulting in highly pathogenic CA-MRSA clones. Worldwide, many strains of *S.aureus* were already resistant to all antibiotics except vancomycin and thus the organism has progressed one step closer to becoming an unstoppable killer (Shakibaie et al., 2002).

Besides the molecular differences that emerged between MSSA/MRSA and PVL negative and positive *S.aureus* clinical isolates, a morphological difference was detected between the tested strains in this study. The majority of MRSA and PVL positive strains appeared as large orange-pigmented colonies on mannitol salt agar, while MSSA mainly appeared as white non-pigmented small colonies. The golden hue was due to the production of carotenoids (isoprenoid lipids) during the stationary phase of growth (Hammond & White, 1970). Carotenoid pigments are considered as a key virulence factor that protect *S. aureus* from the phospholipases present in macrophages and polymorphonuclear neutrophils that hydrolyze phospholipids to produce fatty acids (Chamberlain et al., 1991), thus resisting oxidant-based clearance mechanisms of the host innate immune system (Liu et al., 2005). The simultaneous occurrence of carotenoids and PVL gene in a *S.aureus* strain

increases the virulence of the strain and facilitates its emergence and predominance in the community.

Identification of the *S.aureus* strains using the API system revealed that the API numerical profile of the PVL positive isolates was different from that of the PVL negative isolates. The majority of the PVL negative isolates were capable of fermenting lactose as a carbohydrate source. This additionally, indicates that PVL positive clinical isolates are distinct and separately emerging species. This was in harmony with the published data, which indicated that only the newly emerging CA-MRSA possessed SCC*mec* type IV or V, and harbored the *mecA* and the PVL genes (Kaneko & Kamio, 2004 ; McClure et al., 2006). The use of the Biolog system however, didn't help in revealing any distinct predictable association between biotyping and genotyping, and the phenotypic profiles didn't reveal any distinct predictable pattern. Despite the fact that no other bacterial identification system has as many number of tests as that supplied by the Biolog and that this system remains a valuable tool for the comparisons of microbial communities, the results obtained should be always viewed cautiously (Klingler et al., 1992 ; Heerden et al., 2002). Further refinements in the software are necessary to enable the Biolog system to compete with other systems. Finally, the system was not designed to serve as a primary method for identifying staphylococci in the clinical laboratory (Miller et al., 1993 ; Miller & Roden, 1991).

In conclusion, our study has revealed that the PVL genes are carried by a relatively considerate number of *S.aureus* clinical isolates recovered from patients throughout Lebanon. These isolates were mainly associated with wounds and skin infections, thus asserting that PVL appears as a possible virulence factor related with necrotic lesions of the skin and subcutaneous tissues like furuncles. The PVL positive isolates were from

diverse genetic backgrounds, with a predominance association with CA-MRSA. Currently, the presence of PVL positive *S.aureus* isolates was not detected within hospitals. Nevertheless, close surveillance of these strains is essential to monitor their spread, antimicrobial resistance profiles and association with various diseases. Based on our findings, vancomycin and linezolid were the most efficient antimicrobials agents to treat staphylococcal infections whether they harbored the PVL gene or not.

Finally, experimental comparisons of isogenic PVL-positive and PVL-negative isolates in relevant animals may lead to specific therapeutic approaches targeting PVL in severe PVL-related staphylococcal syndromes and whether a vaccine against PVL could eliminate PVL-positive invasive disease. A better knowledge of the epidemiology, an understanding of the mechanisms involved and improved detection techniques are necessary.

CONCLUSION

➢ A considerable number of *S.aureus* isolates (45%) harbored the PVL gene which is in contrast with a large number of studies that suggest that the PVL prevalence does not exceed 5%. This discrepancy in results might be due to the difference in geographic areas and patients selection. The majority of PVL positive isolates (89.2%) were MRSA belonging to SCC*mec* type IV, thus proving furthermore that PVL can be considered as a stable marker of CA-MRSA worldwide.

➢ *hlg* and PVL form a bi-component synergohymenotropic toxins that result in cell's pore formation. The occurrence of *hlg* gene was detected in 91% of *S.aureus* isolates tested, while most studies reveal that the occurrence of *hlg* gene surpasses 99% of *S.aureus* isolates.

➢ Superficial sites of infections such as wounds were the most common manifestation of staphylococcal infections (42% of all sites of infection), in which 52.9% were PVL positive.

➢ PVL negative *S.aureus* multiple resistant strains were dominant among the tested strains.

➢ Oxacillin and oxytetracycline were found to be the least effective drugs with a percentage of resistance exceeding 30% and 45%, respectively.

➢ Vancomycin and linezolid were the most probably effective antimicrobials against staphylococcal infections regardless whether they harbored the PVL gene or not, with less than 3% resistance.

➢ The combination of carotenoids and PVL gene in a *S.aureus* isolate increases the virulence of the strain and facilitates its widespread distribution in the community.

➢ API numerical profiles were found to be different when compared in PVL positive and negative *S.aureus* isolates. To the extent of our knowledge, this is the first report that highlights the differences based on API numerical profiles between staphylococcal isolates. PVL positive and negative isolates were from diverse genetic backgrounds.

➢ The use of Biolog identification system didn't reveal any predictable phenotypic pattern that distinguishes between PVL positive and negative strains. Although this system is considered as a valuable tool for the identification of microbial species, its results should be read cautiously.

➢ The occurrence of the PVL gene is closely associated with CA-MRSA with 81.5% of the PVL positive isolates being CA-MRSA and since CA-MRSA infections are becoming more widespread, it is of major importance and necessity to establish surveillance programs by health care systems in order to determine the extent of CA-MRSA and PVL gene propagation in the Lebanese community and to monitor their antimicrobial resistance profiles and their association with diseases.

ANNEX I

Basic demographics of the *S. aureus* clinical isolates.

ANNEX

Table 1a. Basic demographics of the AUH PVL negative *S.aureus* clinical isolates.

PVL presence was detected with a duplex PCR amplification.
Hemolysis is obtained with the observation of clear zones around the colony on the blood based agar. Y= hemolytic and N= non-hemolytic.
MRSA is obtained with the Slidex MRSA kit. N= MSSA, Y= MRSA
Gender: F= Female, M= Male

	Hemolysis	MRSA	Gender	Site of infection	Date	Age
A3	N	Y	F	wound	7/4/2006	63
A36	N	Y	M	wound	9/6/2000	63
A24	Y	Y	M	brain	8/4/2002	58
A37	N	Y	M	urine	14/6/2000	67
A38	N	Y	M	wound	17/6/2000	74
A44	N	Y	M	wound	13/7/2001	30
A2	Y	Y	M	wound	3/7/2006	58
A45	N	Y	M	nasal	15/11/2001	22
A18	Y	Y	M	sputum	17/2/2002	87
A19	Y	Y	M	wound	18/2/2002	42
A25	N	Y	M	DTA	9/4/2002	48
A6	N	Y	M	pus	10/12/2005	7M
A14	Y	N	M	pus	12/12/2005	
A15	Y	N	M	sputum	13/12/2005	83
A12	Y	N	M	abcess	29/11/2005	16
A9	Y	N	F	pus	29/11/2005	52
A11	Y	N	F	pus	30/10/2005	35
A34	Y	Y	F	neck mucus	15/4/2002	31
A54	Y	Y	M	wound	25/4/2000	3 M
A59	Y	Y	M	wound	19/5/2000	69
A61	Y	Y	M	wound	21/5/2000	69
A63	Y	Y	M	wound	23/5/2000	65
A64	Y	Y	M	tissue	2/6/2000	69

A72	Y	N	F	pus	26/10/2006	36
A73	Y	N	M	wound	23/10/2006	9
A74	Y	N	M	joint fluid	25/10/2006	77
A75	Y	N	M	sputum	28/10/2006	62
A79	Y	N	M	blood	27/10/06	80
A80	Y	N	F	urine	11/11/2006	65
A81	Y	N	M	DTA	11/11/2006	18
A82	Y	N	F	Abscess	9/11/2006	30
A83	Y	N	M	wound	11/11/2006	50
A85	Y	N	F	eye	14/11/06	72
A86	Y	N	F	DTA	12/11/2006	77
A87	Y	N	M	wound	15/11/06	69
A88	Y	N	M	wound	18/11/06	65
A89	Y	N	F	wound	22/11/06	26
A90	Y	N	M	cath	2/12/2006	13
A91	Y	N	M	DTA	4/12/2006	80
A92	Y	N	M	wound	5/12/2006	28
A93	Y	N	F	pus	5/12/2006	28
A94	Y	N	F	blood	6/12/2006	1
A95	Y	N	M	wound	2/1/2007	73
A97	Y	N	M	DTA	12/1/2007	57
A99	Y	N	F	pus	16/1/07	41
A100	Y	N	M	pus	19/1/07	75

Table 1b: Basic demographics of AUH PVL positive *S.aureus* clinical isolates

	Hemolysis	MRSA	Gender	Site of infection	Date	Age
A43	N	Y	M	wound	1/7/2001	53
A50	N	Y	M	wound	1/2/2002	78
A29	Y	Y	M	wound	3/6/2002	4
A17	Y	Y	F	abcess	4/2/2002	34
A21	Y	Y	M	abcess	4/3/2002	31
A20	N	Y	M	DTA	4/3/2002	50
A30	N	Y	F	wound	5/6/2002	57
A26	Y	Y	F	abcess	7/4/2002	62
A4	Y	Y	M	abcess	6/3/2006	41
A39	N	Y	M	gall bladder	30/6/2000	87
A40	N	Y	M	pus	23/6/2001	68
A46	N	Y	F	catheter	25/11/2001	89

A47	N	Y	F	wound	25/11/2001	41
A41	N	Y	F	wound	26/6/2001	27
A42	N	Y	M	blood	29/6/2001	72
A48	Y	Y	M	wound	31/12/2001	82
A22	Y	Y	F	abcess	13/3/2002	29
A35	N	Y	M	wound	15/4/2002	21
A27	Y	Y	M	wound	16/4/2002	18
A23	N	Y	M	wound	19/3/2002	1M
A33	Y	Y	M	wound	19/8/2002	35
A31	Y	Y	M	wound	21/7/2002	35
A49	N	Y	M	DTA	26/1/2002	72
A28	Y	Y	M	DTA	28/4/2002	60
A32	Y	Y	F	wound	29/7/2002	52
A16	N	Y	M	wound	30/1/2002	34
A8	Y	Y	F	pus	29/11/2005	11
A7	Y	Y	M	wound	29/11/2005	47
A1	Y	Y	F	wound	17/7/2006	1
A5	Y	Y	M	wound	22/1/2006	16
A13	Y	N	F	pue	11/12/2005	48
A10	Y	N	M	fluid	21/11/2005	46
A51	Y	Y	F	wound	25/2/2000	42
A52	Y	Y	F	wound	30/3/2000	38
A53	Y	Y	F	wound	25/4/2000	63
A57	Y	Y	F	eye	14/5/2000	70
A58	Y	Y	F	pus	13/5/2000	27
A65	Y	Y	F	wound	7/6/2000	24
A55	Y	Y	M	DTA	25/4/2000	74
A56	Y	Y	M	wound	10/5/2000	21
A60	Y	Y	M	wound	21/5/2000	1
A62	Y	Y	M	pus	22/5/2000	46
A70	Y	Y	F	wound	27/10/2006	18
A71	Y	Y	F	wound	17/10/2006	2
A66	Y	Y	M		4/9/2006	71
A67	Y	Y	M	blood	9/9/2006	60
A68	Y	Y	M	cyst	11/9/2006	20
A69	Y	Y	M	wound	9/10/2006	58
A76	Y	Y	F	pus	7/11/2006	68
A77	Y	Y	F	sputum	10/11/2006	59
A78	Y	N	F	wound	4/12/2006	85
A84	Y	N	M	DTA	10/11/2006	80
A96	Y	N	M	abscess	13/1/07	11
A98	Y	N	M	BRW	16/1/07	65
A101	Y	N	M	abscess	22/1/07	50
A102	Y	Y	F	abscess	21/1/07	20

Table 2a. Basic demographics of St George PVL negative *S.aureus* isolates.

	Site of infection	Coagulase	Date	Hemolysis	MRSA
G1	Blood	+	2006	N	N
G2	Wound	+	2006	Y	Y
G3	Wound	+	2006	Y	Y
G7	Sputum	+	2006	Y	Y
G8	Wound	+	2006	Y	Y
G9	Wound	+	2006	N	Y
G10	Blood	+	2006	Y	N
G11	Stool	+	2006	Y	N
G12	Stool	+	2006	Y	Y
G13	Stool	+	2006	Y	N
G14	Pus	+	2006	Y	Y
G15	Urine	+	2006	Y	N
G17	---	+	2006	Y	Y
G18	---	+	2006	N	N

Table 2b. Basic demographics of St George PVL positive *S.aureus* isolates.

	Site of infection	Coagulase	Hemolysis	MRSA	Date
G4	Wound	+	Y	Y	2006
G5	Nose	+	Y	N	2006
G6	Blood	+	Y	Y	2006
G16	Pus	+	Y	Y	2006

Table 3a. Basic demographics of Maounat PVL negative *S.aureus* samples.

#	Date	Gender	Site of infection	Hemolysis	MRSA
M1	15-03-06	---	---	Y	Y
M 2	15-03-06	---	---	N	Y
M 4	15-03-06	---	---	Y	N
M 7	26-07-06	M	Blood	Y	N
M 8	29-07-06	F	Breast	Y	N
M9	29-07-06	F	Urine	Y	N
M10	29-07-06	F	Pus	N	Y
M11	29-07-06	M	Nasal	Y	Y
M12	29-07-06	M	Throat Swab	Y	N

M14	7/8/2006	F	Hand	Y	N
M15	7/8/2006	M	Ear Swab	Y	N
M16	31-08-06	M	Blood	Y	N
M17	31-08-06	M	Foot	Y	N
M18	15-09-06	M	Sputum	N	N
M19	15-09-06	F	Stool	Y	N
M20	15-09-06	M	Collection	N	N
M21	18-09-06	M	Wound	Y	N
M22	18-09-06	M	Throat Swab	N	Y
M24	2/10/2006	M	Pus	N	Y
M25	2/10/2006	F	Throat Swab	N	N

Table 3b. Basic demographics of Maounat PVL positive *S.aureus* isolates.

#	Date	Gender	Site of infection	Hemolysis	MRSA
M3	15-03-06	---	---	N	N
M5	15-03-06	---	---	N	Y
M6	15-03-06	---	---	Y	N
M13	8/8/2006	M	Blood	Y	N
M23	2/10/2006	F	Breast	N	Y

ANNEX II

Details of the API numerical profiles.

Table 4 : Details of the API numerical profiles.

	API numerical profile
Profile I	6336153
Profile II	6736153
Profile III	6734153
Profile IV	6336053
Profile V	6736152
Profile VI	6736053
Profile VII	6736151

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