DEVELOPMENT OF A THERMOSTABILIZED MULTIPLEX PCR ASSAY FOR THE RAPID DETECTION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

by

HASSANAIN I. AL-TALIB

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

MARCH 2010

DEDICATIONS

This thesis is dedicated to my mother and my wife and daughters Maryam and Sara, without whose love and support this endeavor would not have been possible.

ACKNOWLEDGEMENT

Upon the production of this dissertation I would like to express my deepest gratitude and sincere appreciation to my project supervisor, Prof. M. Ravichandran who gives me support, invaluable guidance, advice, encouragement and patience throughout the entire progress of this project. His dedication and critical reading are highly appreciated. My thanks also go to my co-supervisors, Dr. Habsah Hasan, Head of Department of Medical Microbiology and Parasitlogy, and Assoc. Prof. Dr. Karim A. Al-Jashamy from Management & Science University. I am very grateful towards their constructive suggestions and unsparing assistance.

I am most grateful to Dr. Chan Yean Yean for valuable discussions, advice, co-operation, and especially for patience and guidance in my process of learning scientific writing. My sincere thanks also goes to Department of Microbiology and Parasitology, Hospital Universiti Sains Malaysia (HUSM) Institute of Medical Research (IMR) Malaysia for providing the clinical isolates as gifts, which were very useful for this study.

I appreciate the help that I received from kak Rosliza Abd Rahman, Mr. Mohd Nadzri Abu Yazid, kak Noral, kak Elly Fatmawatie, Mr. Ishak Hj. Md Isa and the entire Medical laboratory technologists in Dept. of Medical Microbiology and Parasitology.

I also would like to take this opportunity to acknowledge and offer my warmest appreciation to my colleagues Lee Su Yin, Kurunathan, Balqis Kamarudin, Elina, Tan Gim Cheong, Chua Ang Lim, Chandrika and all the staffs in the research laboratory for their immeasurable assistance, support, cooperation and encouragement to me throughout the research.

This work was funded by the Short-term grant (304/PPSP/6131535) from Universiti Sains Malaysia. We are grateful to Institute for postgraduate studies, Universiti Sains Malaysia for their Fellowship support.

Last but not least, my deepest gratitude to my family, especially my mother, my wife, my brothers and my daughters Maryam and Sara also to my sister for the love, concern, encouragement, support and never-ending patience. I wish them be well and happy always. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

%	Percentage
~	Approximately
µg	Micro gram
µl	Micro liter
µM	Micro Molar
β	Beta
<	Less than
≤	Equal and/or less than
>	More than
≥	Equal and/or more than
λ	Lambda (wavelength)

LIST OF ABBREVIATIONS

A	Adenosine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
CFU	Colony forming unit
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
e.g.	*Evempli gratia* or for example
EDTA	Ethylenediamine tetraacetic acid
et al.	*Et alii*
g	Gravity
G	Guanine
g	Gram
i.e.	*Id est* or that is
kb	Kilobase
mg	Miligram
MIC	Minimal inhibitory concentration
min	Minute
m	Mililiter
mM	Milimolar
N	Normal
NCBI	National Centre of Biotechnology Information

ng	nanogram
CLSI	Clinical and Laboratory Standards Institute
°C	Degree Celcius
PCR	Polymerase chain reaction
pg	Pico gram
pmole	Pico mole
rpm	Revolution per minute
T	Thymine
Ta	Annealing temperature
Taq	*Thermus aquaticus*
Tm	Melting temperature
U	Unit
V	Volt
vol.	Volume
wt.	Weight
X	Times or multiplication
PFGE	Pulse field gel electrophoresis
S	Susceptible
UV	Ultra violet
n	Nano
p	Plasmid

LIST OF APPENDICES

PEMBANGUNAN UJIAN REAKSI BERANTAI POLIMERASI BERGANDA STABIL SUHU UNTUK PENGESANAN PANTAS *STAPHYLOCOCCUS AUREUS* YANG RINTANG METHICILLIN

Abstrak

Staphylococcus aureus rintang methicillin (MRSA) bertanggungjawab terhadap kebanyakan jangkitan nosokomial dan komuniti. Ujian kultur konvensional mengambil masa selama dua hingga lima hari untuk menghasilkan maklumat penuh mengenai organisma dan pola kerintangan antibiotiknya. Oleh itu, kajian ini bertujuan untuk membangunkan ujian reaksi berantai polimerasi berganda untuk pengesanan MRSA dengan pantas. Ujian ini akan mengesan lima gen iaitu 16S rRNA gen dari genus *Staphylococcus*, *fem*A *Staphylococcus aureus*, *mec*A yang mengekod rintangan methicillin, *luk*S yang mengekod pengeluaran leukosidin Panton-Valentine (PVL), sitotoksin nekrosis, dan satu gen kawalan dalaman secara serentak. Pasangan primer yang unik dan khusus telah direka untuk mengamplifikasi lima gen dengan produk reaksi berantai polimerasi pada julat 151 hingga 759 bp. Primer yang spesifik disahkan berdasarkan urutan jujukan DNA produk reaksi berantai polimerasi berganda dan analisa Blast. Kepekaan dan kekhususan ujian polimerasi berganda ini telah dibandingkan dengan kaedah kultur konvensional. Reaksi berantai polimerasi berganda ini dijadikan stabil- suhu dan ujian pantas kestabilan telah dinilai pada suhu bilik, 37°C dan 10°C. Ujian kepekaan analitikal pada peringkat DNA didapati adalah 10 ng.

Analisa 34 strain rujukan *Staphylococcus* dan strain bukan *Staphylococcus* terhadap ujian menunjukkan keputusan 100% spesifik. Campuran reaksi berantai polimerasi berganda yang disimpan pada 10°C didapati stabil sehingga dua setengah tahun menurut ujian pantas kestabilan. Kejituan diagnostik ditentukan dengan menggunakan sejumlah 231 sampel swab nasal isolat klinikal. Daripada 231 swab tersebut, hanya 207 menunjukkan pertumbuhan positif untuk *Staphylococcus*. Daripada 207 isolat *Staphylococcus*, 59 disahkan *Staphylococcus aureus*, manakala 148 adalah CoNS. Daripada 59 isolat *Staphylococcus*, 14 didapati rintang methicillin dan menunjukkan gen *mec*A. Kehadiran kawalan dalaman pada ujian reaksi berantai polimerasi berganda membantu dalam menyingkirkan kes negatif palsu. Reaksi berantai polimerasi berganda adalah tegap dan boleh memberi maklumat mengenai 5 gen yang amat penting untuk mengenalpasti spesis *Staphylococcus* dan pola rintangan methicillin mereka. Reaksi berantai polimerasi berganda yang dibangunkan dalam kajian ini boleh digunakan sebagai alat yang berkesan untuk penyaringan dan pengesanan pembawa MRSA di hospital dan di komuniti.

DEVELOPMENT OF A THERMOSTABILIZED MULTIPLEX PCR ASSAY FOR THE RAPID DETECTION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

ABSTRACT

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for nosocomial and community-acquired infections. The conventional culture test takes 2-5 days to yield complete information of the organism and its antibiotic sensitivity pattern. Hence our present study was focused on developing a multiplex PCR assay for the rapid detection of MRSA. The assay simultaneously detected five genes, namely 16S rRNA of the *Staphylococcus* genus, *fem*A of *S. aureus*, *mec*A that encodes methicillin resistance, *luk*S that encodes production of Panton-Valentine leukocidin (PVL), a necrotizing cytotoxin and one internal control. Unique and specific primer pairs were designed to amplify the 5 genes with the PCR products ranging from 151 to 759 bp. The specificity of the primers was confirmed by DNA sequencing of the multiplex PCR products and BLAST analysis. The sensitivity and specificity of multiplex PCR assay was evaluated against the conventional culture method. The multiplex PCR was thermostabilized and an accelerated stability test was evaluated at room temperature, 37°C and 10°C. The analytical sensitivity of the assay was found to be 10 ng at the DNA level while the analytical specificity was evaluated with 34 reference staphylococci and non-staphylococcal strains and was found to be 100%. The thermostabilized multiplex PCR mix stored at 10°C was stable up to two and a half years by the accelerated stability test. The diagnostic accuracy was determined

using a total of 231 nasal swabs clinical isolates. Among these 231 swabs, only 207 showed positive growth for staphylococci. Of these 207, 59 were found to be *S. aureus* while 148 were coagulase-negative staphylococci. Out of the 59 *S. aureus* isolates, 14 were found to be resistant to methicillin and expressed *mec*A gene. The presence of an internal control in the multiplex PCR assay helped to rule out false negative cases. The multiplex PCR assay is robust and can give information about the 5 genes that are essential for the identification of the *Staphylococcus* species and their methicillin resistance pattern. The PCR assay developed in this study can be used as an effective tool for screening and diagnosis of MRSA nasal carrier in hospitals and community.

CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

1.1.1 History and significance of *Staphylococcus*

Genus *Staphylococcus* is one of the most common human pathogens and is capable of causing numerous and serious infections. Although primary *S. aureus* infections are not common, a great deal of the virulence from this organism occurs through cross-infection by spread from patient to patient in hospitals and other institutional settings. In contrast, healthy individuals can be carriers of the organism (Mainous *et al.*, 2006). Nosocomial infections cause a significant crisis for health and economics worldwide. A nosocomial infection is defined as an infection acquired in hospital, and which is not in the incubation phase on the patient's admission to hospital (Garner *et al.*, 1988). Any microorganism including bacteria, parasites, fungi, or viruses can cause nosocomial infections, but bacteria are the most prevalent organisms. Staphylococci were among most common Gram-positive bacteria that causes nosocomial infections (Harbarth *et al.*, 2008). *S. aureus*, in particular, is a leading cause of diseases ranging from mild skin and soft tissue infections to life-threatening illnesses, such as deep postsurgical infections, septicemia, and toxic shock syndrome (Zhang *et al.*, 2004). Risk factors that predispose colonization or infection with multi-resistant species including *S.aureus* are advanced age, severity of illness, inter-institutional transfer, prolonged hospital stay, gastrointestinal surgery, transplantation, exposure to medical devices and exposure to broad-spectrum antibiotics (Safdar and Maki, 2002). Strains of *S. aureus* resistant to all beta-lactam antibiotics, known as methicillin-resistant *S. aureus* (MRSA) causing considerable morbidity and mortality in hospitals. A low-

affinity penicillin-binding protein (PBP2′ or PBP2a) is produced by MRSA strains in addition to the normal PBPs. This altered PBP allows cell wall formation in the presence of antibiotic concentrations which inactivate other PBPs (Chapin and Musgnug, 2004). The gene encoding PBP2a has been named *mec*A and was found in all staphylococci sharing methicillin resistance mediated through PBP2a. The presence of *mec*A gene and expression of low-affinity PBP is considered the most important mechanism of methicillin resistance in *S. aureus* and other staphylococci (Barski *et al.*, 1996). In addition to all beta-lactam antibiotics MRSA is resistant also to, cephalosporins and staphylococcal penicillins (Berger-Bachi, 1999). The epidemiology of MRSA is changing constantly, the rates of methicillin resistance differ markedly among countries and range from < 1 percent in Scandinavia to >30 percent in southern European countries (Prasad *et al.*, 2000).

1.1.1.1 Historical review of the emergence of *Staphylococcus aureus* and antimicrobial resistance

The history of the emergence of resistance and changes in the epidemiology of infection with *S. auerus* is depicted in (Table 1.1).

Table 1.1. Emergence of antimicrobial resistance and changing epidemiology of *S. aureus*

Date	Events
1880	Sir Alexander Ogston discovered *S. aureus* in Aberdeen Scotland
1928	Alexander Fleming discovered penicillin, using strains of Staphylococcus
1939	There were strains of Staphylococcus resistant to penicillin
1940	Hospitals in the United Kingdom and the United States reported that 50% of *S. aureus* was resistant to penicillin
1942	Penicillin used in humans
1943	Beta-lactamase detected in *S. aureus*
1950s	Penicillin resistance common in hospital strains of *S. aureus*
1957	Methicillin released in UK
1961	MRSA reported in UK
1960s	Increasing reports of MRSA, multiple hospital outbreaks
1966	First MRSA reported in Australia
1970s	Penicillin resistance common in community MSSA
1976	Outbreaks of gentamycin resistant MRSA in Melbourne, UK
Late 1970s	Multiple MRSA outbreaks throughout world
1981	Extensive outbreaks of Gentamycin resistance MRSA in Ireland, USA, Australia
1980s	MRSA becomes endemic in Australia, USA, other countries
1990	MRSA endemic in most hospitals throughout the world
1998	Emergence of strains of *S. aureus* resistance to vancomycin, in Japan, then USA

Note: Adapted and slightly modified from reference (Gosbell, 2003) appeared as table 1.1 and page 2 in the source of original.

1.2 CLASSIFICATION OF STAPHYLOCOCCUS SPECIES

The main classification of *staphylococcus* is by presence or absence of coagulase production. Coagulase is a protein product, which is an enzyme that causes clot formation.

1.2.1 Coagulase-positive Staphylococci

The Coagulase-positive *Staphylococcus* included *S. aureus* and *S. intermedius*, *S. delphini*, *S. lutrae* and some strain of *S. hyicus*. Coagulase-positive pathogens have the ability to produce coagulase, a protein product which is an enzyme that causes clot formation while most other *Staphylococcus* species are coagulase-negative. However, the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase. *S. aureus* is catalase positive which produce the enzyme catalase and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish Staphylococci from Enterococci and Streptococci.

1.2.2 Coagulase-negative Staphylococci

Coagulase-negative staphylococci (CoNS) are common colonizers of the human skin and the most frequent constituent of the normal flora at this site (Roth and James, 1988). Once considered a relatively virulent and probably a contaminant when isolated from a clinical specimen, these organisms have become increasingly recognized as agents of clinically significant nosocomial bloodstream infections. There are 31 species currently recognized in the genus *staphylococcus*, which are members of the Micrococcaceae family (Kloos and Bannerman, 1994). Although at least 18 species

have been isolated from human skin (Hamory *et al.*, 1987), *S. epidermidis* accounts for more than half of resident staphylococci (Leeming *et al.*, 1984) with extensive distribution over the body surface. In terms of clinical isolates, *S. epidermidis* is clearly predominant, comprising more than 75 percent of CoNS in clinical specimens (Pfaller and Herwaldt, 1988), this is perhaps due to its sheer numbers on the skin surface, although it may possess virulence determinants that other CoNS lack (Haveri *et al.*, 2007). Other clinically significant species include *S. saprophyticus* that is part of the normal vaginal flora is predominantly implicated in genitourinary tract infections in young adult women (Wallmark *et al.*, 1978), while *S. hominis*, *S. haemolyticus*, *S. warneri*, and *S. simulans* have been more rarely isolated as pathogens (Pfaller and Herwaldt, 1988). *S. lugdunensis* has increasingly been recognized as a cause of invasive infections that include endocarditis, osteomyelitis, and sepsis (Ebright *et al.*, 2004).

1.3 *STAPHYLOCOCCUS AUREUS* STRUCTURE, VIRULENCE FACTORS, AND PATHOGENESIS

1.3.1 Cell wall

The outermost layers of pathogens are important in the infection process (Anthony and Hill, 1988). Most *S. aureus* isolates are covered by a polysaccharide capsule. Based on capsular polysaccharides *S. aureus* can be classified into eleven different serotypes. Beneath the capsule *S. aureus* harbors a typical Gram positive cell wall (Giesbrecht *et al.*, 1998). The Gram positive cell wall has a thicker and highly cross linked peptidoglycan layer than Gram negative and it lacks the outer membrane (Beveridge, 1999, van Wely *et al.*, 2001) (Figure 1.1).

The peptidoglycan consists of glycan strands of N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) disaccharides, cross linked by tetrapeptides consisting of L-alanine, D-glutamine, L-lysine, and D-alanine. In *S. aureus,* a pentaglysine inter-bridge links the tetrapeptide units of adjacent glycan strands. *S. aureus* produces four penicillin-binding proteins (PBPs) , PBP1-4, involved in the cell wall peptidoglycan assembly (Labischinski, 1992). PBP2 is a bifunctional protein which, in addition to transpeptidase activity, also acts as transglycosylase (Goffin and Ghuysen, 1998). PBPs bind effectively to beta-lactam antibiotics, and in the presence of these agents, the cell wall assembly is discontinued.

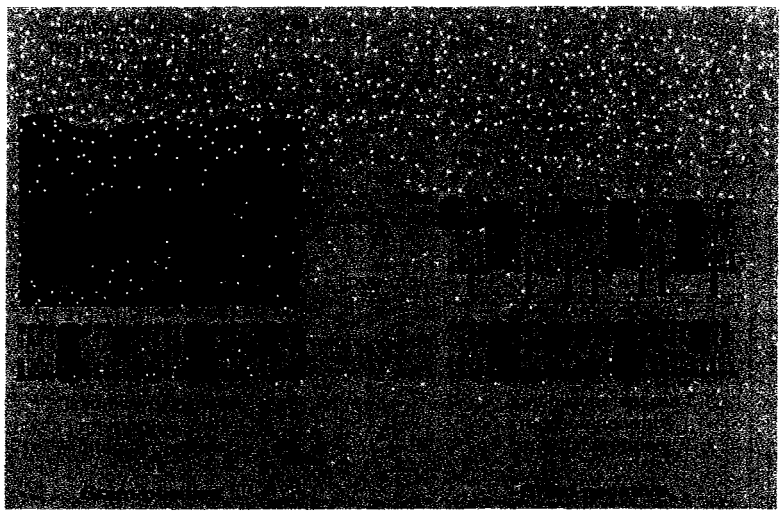

Figure 1.1. Composition of cell wall of Gram-positive and Gram negative bacteria. Note: Adapted from reference (Cedric Mims, 2004) appeared as table 1.1 and page 2 in the source of original.

1.3.2 Genome of *S. aureus*

The genomic positions of various genetic markers of *S.aureus* have been localized by creating physical maps of the genome. The development of *S. aureus* chromosomal maps began through definition of three linkage groups consisting of nine auxotrophic markers and a novobiocin resistance marker on *S. aureus* NCTC 8325 (Pattee and Neveln, 1975). By inclusion of a large number of additional markers, and the use of pulsed field gel electrophoresis (PFGE) and subsequent hybridization with available probes, the physical map of *S. aureus* NCTC 8325 continued to be more and more precise (Iandolo, 2000) (Figure 1.2). However, until data from genome sequencing became available, the mutual distances between genetic markers within each PFGE fragment will be unknown.

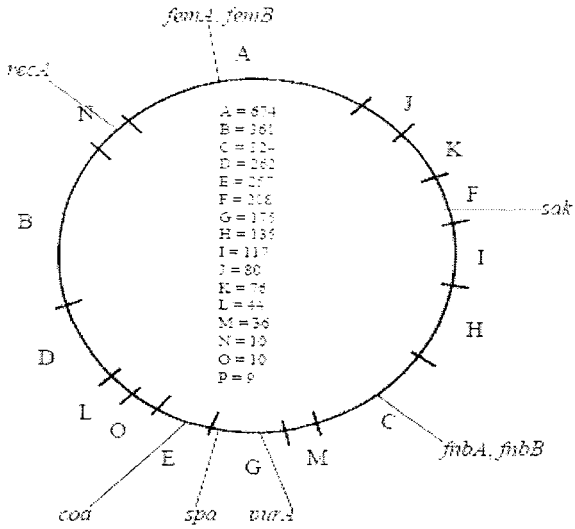

Figure 1.2. *S. aureus* NCTC 8325 physical map. Adapted from (Saara, 2002). *SmaI* restriction fragments A-P, their sizes in kilobase pairs, and examples of identified genetic markers.

1.3.3 Virulence factors

The pathogenicity associated with *S. aureus* can be attributed to a number of virulence factors, such as enterotoxins, cytolytic toxin, and cellular components like protein A. According to their biological function, the virulence factors can be divided into three groups: those involved in adhesion, in host defense evasion, and in tissue penetration (Table1.2).

1.3.3.1 Enterotoxins

Staphylococcal enterotoxins are heat stable exotoxins that cause diarrhea and vomiting in humans. Eight serological distinct enterotoxins (A-E and G-I) have been identified

(Wieneke *et al.*, 1993). These toxins are produced by 30% to 50% of *S. aureus* isolates. These toxins along with toxic shock syndrome toxin-1 (TSST-1), are superantigens having the ability to activate a strong overactive immune response. TSST-1 causes nearly all cases of menstruating associated TSS. TSST-1 is a superantigen stimulating T cell proliferation and the subsequent production of a large concentration of cytokines that are responsible for the symptoms (Ladhani *et al.*, 1999).

1.3.3.2 Cytolytic toxins

S. aureus produces other extracellular proteins that affect red blood cells and leukocytes. There are four types of hemolysins: alpha, beta, gamma and delta, α-Hemolysin is dermonecrotic and neurotoxic, lyses erythrocytes, can damage platelets, macrophages and causes sever tissue damage (Bhakdi and Tranum-Jensen, 1991). β-Hemolysin, acts on sphengomyelin in the plasma membrane of erythrocytes. **δ**-Hemolysin, although found in a higher percentage of *S. aureus* and CoNS, is considered less toxic to cell surface than other hemolysins (Dinges *et al.*, 2000). γ-Hemolysin is often only found associated with Panton-Valentine leucocidin (PVL). Staphylococcal leucocidin, PVL, is an exotoxin that is lethal to polymorphonuclear leukocytes. It has been implicated as contributing to the invasiveness of the organism by suppressing phagocytosis and has been associated with sever cutaneous infections and necrotizing pneumonia. Although produced by relatively few strains of *S. aureus*, it has been associated with some cases of community-acquired staphylococcal infections (Dinges *et al.*, 2000).

1.3.3.3 Enzymes

Several enzymes are produced by staphylococci like coagulase, protease, hyaluronidase and lipase. Coagulase is produced mainly by *S. aureus*, although the exact role of coagulase in pathogenicity remains uncertain, it is considered a virulence marker (Siboo *et al.*, 2001). Many strains of *S. aureus* produce hyaluronidase. This enzyme hydrolyzes hyaluronic acid present in the intracellular ground substance that makes up connective tissues, permitting the easy spread of bacteria during infection. Lipase is produced by both coagulase positive and coagulase- negative staphylococci. Lipases acts on lipid present on the surface of the skin, particularly fats and oil secreted by sebaceous glands. Protease, lipase and hyaluronidase are capable of destroying tissue and may facilitate the spread of infection to adjoining tissues.

1.3.3.4 Protein A

Protein A is one of several cellular components that have been identified in the cell wall of *S. aureus*. The most important role of protein A in infections caused by *S. aureus* is its ability to bind the Fc portion of immunoglobulin G (IgG), which eventually block phagocytosis (Harrison, 2007).

Genes for *S. aureus* virulence factors may reside in plasmids, bacteriophages, transposons, or pathogenicity islands of the chromosome. Recent sequencing of two *S. aureus* strains revealed three new classes of pathogenicity islands: TSST family islands, exotoxin islands, and enterotoxin islands. A considerable number of putative virulence genes were also identified (Kuroda *et al.*, 2001)

Table1.2. *Stapylococcus aureus* virulence factors.

Virulence factor	Gene	Regulation system	Proposed virulence function	Production phase
Enterotoxin A	*ent*A	agr	Host defence evasion	All
Enterotoxins B-E, G-J	*ent*B-E, *ent*G-J	agr	Host defence evasion	Post exponential
Hemolysin	*hlg*	agr	Tissue penetration	Post exponential
Panton-Valentine leucocidin	*luk*F-PV, *luk*S - PV	n.d.	Host defence evasion	Post exponential
Coagulase	*coa*	agr, sae	Attachment	Exponential
Serine protease	*spr*	agr, sar	Host defence evasion	Post exponential
Hyaluronidase	*hys*A, *hal*	agr	Tissue penetration	Post exponential
Lipase	*geh*	agr, sar	Host defence evasion	Post exponential
Protein A	*spa*	agr, sarA, sarS, sae	Host defence evasion, attachment	Exponential
Clumping factor	*clf*A	sar	Attachment	Exponential
Leucocidin R	*luk*F-R, *luk*S-R	agr	Host defence evasion	n.d.
Fibrinogen binding protein A	*fba*A	agr, sar	Attachment	Exponential
Toxic shock syndrome toxin	*tst*	agr, sar	Host defence evasion	Post exponential

Note: Adapted and modified from (Projan, 1997), (table 3-1) (n.d, not determined or not reported in the literature).

1.3.4 Pathogenesis and immune response

Staphylococcal pathogenesis results from various bacterial activities mediated by virulence factors, and from the immunological response by the host. It is commonly thought that bacterial adherence to host tissue is a prerequisite for colonization and infection (Patti *et al.*, 1994). Subsequent survival, growth, and establishment of infection depend on the ability of the bacterium to circumvent host defense. The primary host response is mediated by polymorphonuclear leucocytes (Verdrengh and Tarkowski, 1997), which are attracted by expression of adhesion molecules on endothelial cells. The cell wall components, peptidoglycan and teichoic acids, trigger signaling pathways leading to the release of cytokines (Ellingsen *et al.*, 2002). Leucocytes and other host cellular factors can be destructed by locally acting bacterial toxins. Anti-inflammatory response is also achieved by the staphylococcal extracellular adherence protein, which inhibits the recruitment of host leucocytes by direct interaction with the host adhesive proteins ICAM-1, fibrinogen, and vitronectin (Chavakis *et al.*, 2002). The robust local inflammatory response may lead to the formation of an abscess. Inside an abscess, the bacteria gradually fall into a state of nutritional stress as the density of bacteria increases. At this point the autoinduction of secreted virulence factors could enable the bacteria to break out and spread to new locations (Novick *et al.*, 1995).

In toxin mediated diseases, superantigens bind non-specifically to the major histocompatibility complex II (MHC II) and crosslink it to the variable beta chain of T-lymphocyte. Since the normal route of internalization, processing, and antigen presentation is bypassed, this unspecific binding leads to massive expansion of T-lymphocytes and production of cytokines. Superantigens also induce endotoxin hypersensitivity and bind directly to endothelial surfaces, probably causing capillary leakage through endothelial cell death or intercellular gap formation.

In invasive diseases, such as sepsis and endocarditis, staphylococci must interact with the endothelium. The bacteria can adhere to damaged areas of the endothelium, or directly to the endothelial cell via the adhesin-receptor mechanism or via bridging ligands (Joh *et al.*, 1999). The bacteria may then be phagocytized into endothelial cells and/or reach the underlining tissue (Lowy, 1998). Both endothelial phagocytosis and tissue invasion elicit an inflammatory response leading to the release of IL -1, -6, -8, tumor necrosis factor (TNF), and subsequently gamma interferon. Leucocytes adhere to endothelial cells and increase vascular permeability. Although *S. aureus* is primarily an extracellular pathogen, it may sometimes survive inside non-professional phagocytes, such as fibroblasts, renal cells, and osteoblasts. Intracellular survival may explain the persistent and recurrent nature of certain staphylococcal infections (Proctor *et al.*, 1995). Intracellular staphylococci often appear as small colony variants which have mutations affecting electron transport, and show slowly growing, nonpigmented colonies with reduced production of virulence factors (von Eiff *et al.*, 1997).

1.3.4.1 Infections caused by staphylococcus

Staphylococcus aureus causes a wide variety of diseases, from mild skin infections to severe life threatening systemic infections (Waldvogel, 2000). It is a common cause of skin and subcutaneus infections, including folliculitis, furunculosis, cellulitis, mastitis, and impetigo. Recurrent abscesses of the skin and the subcutaneous tissue may be difficult to treat. The preferable treatment for folliculitis and local abscesses is surgical drainage, whereas cellulitis is usually treated with antimicrobials. Impetigo can range from mild, recurrent infections to a more severe bullous form and to the potentially life-threatening scalded skin syndrome (Ladhani *et al.*, 1999). *S. aureus* is also commonly associated with postoperative wound infections, catheter-related infections, toxic shock syndrome (TSS), and food poisoning. TSS and food poisoning are toxin-mediated diseases. The common, self-limiting, food poisoning is caused by enterotoxins present in contaminated food, and is characterized by nausea, vomiting, headache, and sometimes diarrhea. The symptoms start four to five hours after consumption of contaminated food (Wieneke *et al.*, 1993). TSS, caused by TSST-1, is a potentially fatal condition, most commonly associated with the use of highly absorbent tampons, but also known in non-invasive *S. aureus* infections in children. The symptoms include high fever, rash, desquamation of skin one to two weeks after onset, hypotension, and involvement of multiple organ systems (Dinges *et al.*, 2000). Serious *S. aureus* infections include osteomyelitis, pneumonia, sepsis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, scalded skin syndrome, and sterile site abscesses (Waldvogel, 2000). *S. aureus* pneumonia is rare in a community setting, but fairly common in a hospital setting, especially as a

consequence of influenza in elderly patients (Lowy, 1998). Acute osteomyelitis primarily affects long bones in children, whereas chronic (duration of infection >6 months) osteomyelitis is more common in adults after bacteremia, or as a complication of penetrating wounds (Waldvogel and Papageorgiou, 1980). *S. aureus* sepsis most often originates in a local infection focus such as cellulitis, pneumonia, or a wound, or is related to an intravascular device. Complicated sepsis may hematogenously spread the infection to other organs, such as heart, bone, and joint (Lyytikainen *et al.*, 2002)

1.3.4.2 Staphylococcal nasal carriage

Humans are a natural reservoir of *S. aureus* and the primary ecological niches of *S. aureus* are the anterior nares, although other body sites such as the throat, perineum, groin, and skin may also be colonized. *S. aureus* nasal carriage has been identified as a major risk factor in the development of infections not only in the hospital setting (von Eiff *et al.*, 2001), but in the community as well (Wertheim *et al.*, 2005). Many underlying diseases or conditions such as insulin-dependent diabetes mellitus, continuous ambulatory peritoneal dialysis, intravenous drug abuse, human immunodeficiency virus (HIV) infection, and *S. aureus* skin infections and other skin diseases have been associated with a higher *S. aureus* nasal carriage and subsequent infection rate (Berman *et al.*, 1987, Luzar *et al.*, 1990, Nguyen *et al.*, 1999, Williams *et al.*, 1998). According to cross-sectional studies, a mean carriage rate of 37% was found when investigating the prevalence and incidence of *S. aureus* nasal carriage (Kluytmans *et al.*, 1997). The *S. aureus* nasal carriage rate may also have changed over the years; previous studies have reported higher rates than those published recently (Kluytmans *et al.*, 1997, Wertheim *et al.*, 2005). Nasal carriage patterns differ

between healthy persons, and persistent carriage has been reported in 10-35% of individuals, 20-75% carry *S aureus* intermittently, and 5-50% never carry *S. aureus* (Kluytmans *et al.*, 1997).

The non-carrier state may be attributable to bacterial interference with each other: when the ecological niche is already occupied by other bacteria such as CoNS or *Corynebacterium* species, *S. aureus* does not seem to replace the resident bacterial population (Hu *et al.*, 1995). Persistent carriage is more common in young children than in adults, and the carriage pattern has been reported to change in many persons between age 10-20 (Armstrong-Esther, 1976). In addition, a persistent carriage rate is higher in males than in females, and depends on hormonal status (Eriksen *et al.*, 1995).

Significantly higher numbers of *S. aureus* bacteria have been reported in the nostrils of persistent carriers than in those of intermittent carriers, which results in an increased risk of *S. aureus* infections; elderly healthy persistent carriers had higher amounts of *S. aureus* bacteria than did young carriers (Nouwen *et al.*, 2004). Based on molecular studies, the exchange rate of *S. aureus* strains has been reported to be significantly higher in intermittent carriers than in persistent carriers (VandenBergh *et al.*, 1999). Cespedes and colleagues have developed a mathematical model for investigating the frequency of the simultaneous nasal carriage of multiple strains of *S. aureus* (Cespedes et al., 2005). According to that study, 6.6% of *S. aureus*-colonized individuals carry more than one strain. The presence of more than one strain of *S. aureus* at the same time increases the potential for the horizontal transfer of genes, including virulence determinants or antimicrobial-resistance genes. This may be a problem when a single antibiotic-susceptible isolate, rather than another, more

resistant strain from patients infected with *S. aureus* is only detected. The treatment may therefore be unsuitable.

1.4 ANTIMICROBIAL RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

Staphylococci are intrinsically resistant to broad-spectrum antimicrobial agents and this limits the choice of drugs for treatment. The increased prevalence of staphylococcal pathogens in nosocomial infections worldwide has resulted in antimicrobials being used in greater frequency in hospitals

1.4.1 Mechanisms of antimicrobial resistance

1.4.1.1 Resistance to Beta-lactams

S. aureus can be resistant to Beta-lactams via several mechanisms below:

(i) Production of Beta-lactamase (Penicillinase) conferring resistance to Penicillin

Beta-lactams are known to bind to cell wall enzymes known as PBPs, the cross-linking or transpeptidation reactions take place on the external surface of the cytoplasmic membrane in a reaction catalysed by PBPs. Beta-lactamase The β-lactam antibiotics, inhibit the transpeptidation domain of PBPs, thus interfering with the cross-linking reaction (Stapleton and Taylor, 2002). Without cross-linking of the peptidoglycan, the cell wall becomes mechanically weak, some of the cytoplasmic contents are released and the cell dies (Giesbrecht *et al.*, 1998). There are four PBPs in *S. aureus*, PBP1, PBP2, PBP3, and PBP4, which are essential for cell growth, and have high affinity for most β-lactams.

(ii) Production of an altered Penicillin-binding protein (PBP 2a) conferring resistance to methicillin

The term "methicillin resistance" denotes resistance of *S. aureus* to all β-lactam antibiotics (penicillin, monobacams and carbapenms) due to the presence of an altered PBP in the cell wall known as PBP 2a (Berger-Bachi, 1997). PBP 2a is a 76-kDa and is the product of the gene *mec*A. There is no *mec*A homolog in susceptible strains. Both susceptible and resistant strains of *S. aureus* produce four major PBPs, PBPs 1, 2, 3, and 4 (Chambers, 1997). PBPs catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. β-lactam antibiotics are substrate analogs that covalently bind to the PBP active-site serine. PBPs 1, 2, and 3, have high affinity for most β-lactam antibiotics, are essential for cell growth and for the survival of susceptible strains, and binding of β-lactams by these PBPs is lethal (Chambers and Sachdeva, 1990). *mec*A is highly conserved among staphylococcal species. In methicillin-resistant cells, PBP 2a, with its low affinity for binding β-lactam antibiotics can substitute for the essential functions of high-affinity PBPs at concentrations of antibiotic that are otherwise lethal (Utsui and Yokota, 1985).

(iii) Point mutations in penicillin-binding protein

S. aureus may have a raised methicillin MIC by another mechanism. Point mutations may occur in the penicillin-binding domains of PBPs 1, 2 and 4, with a resultant reduction in affinity for β-lactams (Tomasz *et al.*, 1989). Other study found alterations in PBPs 2 and 4 in resistant mutants passaged in medium containing β-lactams (Berger-Bachi *et al.*, 1986). These altered PBPs bind penicillin more slowly and

released penicillin more rapidly (Chambers *et al.*, 1994). These binding alterations are due to point mutations affecting the penicillin-binding domains (Hackbarth *et al.*, 1995). Over expression of PBPs, especially PBP4 may also result in low-level resistance as more enzyme is unbound and available for cell wall synthesis. These strain have been referred to as MODSA (modified *S. aureus*) (Berger-Bachi, 1997), although some authors classify these strains as also having borderline resistance (Chambers, 1997).

(iv) Production of a novel enzyme with a higher affinity to methicillin or overproduction of beta-lactamase

Borderline oxacillin-resistant *S. aureus* (BORSA) strains exhibit MICs to oxacillin or methicillin just above the CLSI breakpoints (oxacillin MIC 4-8 mg/L) and do not possess the *mec*A determinant (Chambers, 1997). The mechanism of resistance in BORSA is either a higher affinity to methicillin or overproduction of β- lactamase (Massidda *et al.*, 1992).

In the case of BORSA strains possessing "methicillinase", production of this enzyme is inducible. During the investigation of BORSA strains VU94 and 822 two beta-lactamases were detected in the membranes, with molecular weights of 13 and 30 kDa. Both beta-lactamases were detected in the MRSA strain studied, but the susceptible strains possessed only the first enzyme. The 30-kDa β- lactamase proved to be a methicillinase, and it can be one of the main causes of the borderline phenotype of BORSA strains (Keseru *et al.*, 2005).

BORSA strains with overproduction of β- lactamase are *mec*A negative, show high level of β- lactamase activity against benzylpenicillin as well as partial and slow hydrolysis of methicillin, oxacillin and cephalosporins (Kernodle *et al.*, 1989).

1.4.1.2 Resistance to non- Beta lactams

Most antibiotic resistance in *S. aureus* is encoded by plasmids, transposons and insertion sequences, allowing rapid spread of resistance from strain to strain and also from CoNS to *S. aureus*, many antibiotic resistance gene are shared between *S. aureus* and CoNS (Paulsen, 1997). Staphylococcal cassette chromosome *mec* (SCC*mec*) can act as a trap for the integration of other, unrelated resistance determinants (Berger-Bachi, 1997). IS431 *mec*, specially, may serve as a target of integration of other insertion sequences or plasmids flanked by similar insertion sequence elements, Tn 554 can also integrate upstream of *mec*A due to the presence of transposons attachment sites there (Stewart *et al.*, 1994).

1.5 LABORATORY DIAGNOSIS

S.aureus is a Gram positive coccus; it appears as grape-like clusters when viewed through a microscope nonmotile, non-spore forming, and catalase positive aerobic or anaerobic showing hemolytic and large golden-yellow colonies. Colonies produced after 18 to 24 hours of incubation appear cream-colored. Staphylococci are common isolates in the clinical laboratory and are responsible for several suppurative types of infections. The laboratory diagnostics is based on culture and biochemical tests, typical morphology, positive coagulase reaction, fermentation of mannitol and trehalose, and production of heat stabile nuclease (thermonuclease) Tables 1.3 and 1.4. The ability of coagulase to clot plasma is the most widely used method for identification. A four-hour tube coagulase test with reconstituted plasma is definitive, and a slide test for bound coagulase is a means of rapid screening for species identification (Kloos, 1999).

Characteristics		species									
		Staphylococci	Enterococci	Streptococci	Aerococci	Alloiococci	Planococci	Stomatococci	Macrococci	Micrococci	Rothia
Strict aerobe		-	-	-	-	+	+	-	±	+	-
Facultative anaerobe		d	+	+	+	-	-	+	±	-	+
Motility		-	d	-	-	-	+	-	-	-	-
Growth with NaCl agar	5% NaCl	+	+	d	+	+	+	-	+	+	-
	6.5% NaCl	+	+	d	+	+	+	-	+	+	-
	12% NaCl	d	(±)	-	+	ND	+	-	±	d	-
Catalase		+	-	-	-	±	+	±	+	+	±
Benzidine test		+	-	-	-	-	+	+	+	+	+
Anaerobic acid from glucose		d	+	+	(+)	ND	-	+	-	-	+
Lysostaphine (200µg/mL)		-	+	+	+	ND	+	+	-	+	+
Erythromycin (0.4 µg/mL)		+	+	-	ND	ND	ND	ND	+	-	ND
Bacitracin (0.04-U disk)		+	+	d	-	ND	ND	-	+	-	-

Note: Adapted from reference (Murray, 2003) appeared as table : Staphylococcus, Micrococcus, and other catalase positive cocci that grow aerobically. +, 90% or more strains positive; ±, 90% or more strains weakly positive; -, 90% or more strains negative; d, 11% to 89% of strains positive; (), delayed reaction; ND, not determined.

		species						
Test		*S. aureus*	*S. epidermidis*	*S. haemolyticus*	*S. lugdunensis*	*S. saprophyticus*	*S. schleiferi*	*S. simulans*
Colony pigment		+	-	d	d	d	-	-
Staphylocoagulase		+	-	-	-	-	-	+
Clumping factor		+	-	-	(+)	-	+	-
Heat-stable nuclease		+	-	-	-	-	+	-
Alkaline phosphatase		+	+	-	-	-	+	(d)
Pyrrolidonyl arylamidase		-	-	+	+	-	+	+
Ornithine decarboxylase		-	(d)	-	+	-	-	-
Urease		d	+	-	d	+	-	+
β- Galactosidase		-	-	-	-	+	(+)	+
Acetoin production		+	+	+	+	+	+	d
Novobiocin resistance		S	S	S	S	R	S	S
Polymyxin B resistance		S	R	S	S/R	S	S	S
Acid (aerobically from)	D-Trehalose	+	-	+	+	+	d	d
	D-Mannitol	+	-	d	-	d	-	+
	D-Mannose	+	(+)	-	+	-	+	d
	D-Turanose	+	(d)	(d)	(d)	+	-	-
	D-Xylose	-	-	-	-	-	-	-
	D-Cellubiose	-	-	-	-	-	-	-
	Maltose	+	+	+	+	+	-	(±)
	Sucrose	+	+	+	+	+	-	+

Note: Adapted from reference (Murray, 2003) appeared as table : Staphylococcus, Micrococcus, and other catalase positive cocci that grow aerobically. +, 90% or more strains positive; ±, 90% or more strains weakly positive; -, 90% or more strains negative; d, 11% to 89% of strains positive; (), delayed reaction; R, resistant; S, sensitive.