MOLECULAR EPIDEMIOLOGY CHARACTERIZATION OF CLINICALLY IMPORTANT STAPHYLOCOCCUS AUREUS

AMGHALIA M. S. ELMNAFI

FPSK(m) 2009 15
MOLECULAR EPIDEMIOLOGY CHARACTERIZATION OF CLINICALLY IMPORTANT *STAPHYLOCOCCUS AUREUS*

By

AMGHALIA M. S. ELMNAFI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science

November 2009
DEDICATION

To my late mother Saleha.

To my husband Khaled for his affection and constant support.

To my father for his understanding, encouragement and open-mindedness.

To my children Musaab, Israa and Aseel for being a great source of motivation, strength and laughter.

To my sisters, brothers and family in law for their encouragement and moral assistance.
Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

MOLECULAR EPIDEMIOLOGY CHARACTERIZATION OF CLINICALLY IMPORTANT STAPHYLOCOCCUS AUREUS

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Chairman: Mariana Nor Shamsudin, PhD

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Methicillin-resistant *Staphylococcus aureus* (MRSA), a hospital based infection has emerged as a cause of skin infections and invasive infections among healthy adults and children in the community. The present research successfully established molecular characteristics of clinical MRSA isolates to differentiate these strains from methicillin sensitive *Staphylococcus aureus* (MSSA) isolates. These relevant molecular characteristics of local MRSA contribute as initial database of these isolates in order to fully understand the epidemiology, microbiology, and pathophysiology of these infections. A total of 90 isolates from different locations in Malaysia were included in the study.
To investigate the epidemiology of *S. aureus* in Malaysia, two highly reliable typing methods, randomly amplified polymorphic DNA and Rep-PCR were applied to 50 *S. aureus* hospital isolates showed consistent clonal groupings of isolates based on geographical locations. However, MRSA and MSSA strains are clustered together with no differentiation into separate groups or cluster. Further molecular differentiation of isolates was obtained through genotypic profiling 16S ribosomal RNA (rRNA) gene sequence. The clinical isolates were differentiated from the environmental isolates. An important finding of the research is the optimization of molecular methods for simple amplification of various genes useful in epidemiological-linked infection management. These genes include those involved in methicillin-resistant *S. aureus*. By using *S. aureus* strains to be tested as templates, various oligonucleotides primers amplified the 533-bp region of *mecA*, 310-bp region of the *mecR1* penicillin binding domain gene, 318-bp region of the *mecR1* transmembrane domain gene, and 481-bp region of the *mecI* gene. The presence of these genes was confirmed by nucleotide sequence analysis.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk darjah Master Sains

KARAKTERISASI EPIDEMIOLOGY MOLEKUL STAFILOKOKUS AUREUS KLINIKAL PENTING

Oleh

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Untuk menyiasat epidemiologi S. aureus di Malaysia, dua kaedah mengetaip yang amat dipercayai, iaitu penggandaan rawak polimofik DNA dan Rep-PCR, telah dijalankan ke
atas 50 isolat-isolat *S. aureus* hospital dan menunjukkan pengumpulan klon secara konsisten isolat-isolat berdasarkan lokasi geografi. Akan tetapi, jenis-jenis MRSA and MSSA tidak dibezakan kepada kumpulan atau kelompok yang berasingan, malahan dikelompok bersama. Pembezaan molekular lanjut bagi isolat-isolat telah diperolehi melalui pemprofillan genotaip turutan gen ribosom RNA (rRNA) 16S. Isolat-isolat klinikal telah dibezakan daripada isolat-isolat persekitaran.

Penemuan penting dalam penyelidikan ini adalah pengoptimuman kaedah-kaedah molekular mudah untuk mengamplifikasi pelbagai gen yang berguna dalam pengurusan jangkitan yang berkaitan dengan epidemiologi. Gen-gen meliputi gen yang berkaitan dengan *S. aureus* tahan metisilin. Dengan menggunakan jenis-jenis *S. aureus* yang bakal diuji sebagai templat, pelbagai primer oligonukleotida berjaya mengamplifikasi kawasan 533-bp *mecA*, kawasan 310-bp gen domain pengikat penisilin *mecR1*, kawasan 318-bp gen domain transmembran *mecR1*, dan kawasan 481-bp gen *mecI*. Kehadiran gen-gen ini telah disahkan melalui analisis turutan nukleotida.
ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim.

In the name of Allah S.W.T.,

I would like to thank my advisor Assoc. Prof. Dr. Mariana Nor Shamsudin for her guidance, advice and support throughout my work. She has helped me a lot from the beginning of my program at University Putra Malaysia I don’t intend to exaggerate with words. All I want to say is sincere and straight from my heart: "Thank you". I would like also to thank my co-supervisors, Prof. Dr. Raha Abd. Rahim and Assoc. Prof. Dr. Zamberi Sekawi for their help and constructive criticism during my study. Special and very deep thanks to Dr. Naji Al-haj for his big support to finish my work. Many thanks to Miri Hospital, Kuantan Hospital, Seremban Hospital and Hospital University KL for providing the pure bacteria stock culture of *Staphylococcus aureus*. Deep thanks to Mr. Zainan Ahmad Ariffin and Mr. Rahman Mohd. Taib for the technical assistance during this study. Thanks my colleagues and friends, Vasantha Kumari Neela, Wan Somarny, Faizah Jaafar, Halimatun Hamat, MasIdayu Mashan, Nor Farra Alipiah, Lai Suang for their supports. I would like to thank the people libyan arab jamaheria for supporting me financially for three years. Heartful thanks to to my husband Mr. Khaled K. M. Erghibi and my children Musaab, Israa and Aseel Khaled for their understanding and patience. Last but not least, I would like to thank all faculty and staff members in the Department of Medical Microbiology and Parasitology for their company, discussion and help during the period of my study.
This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Sciences.

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Data: 11 February 2010
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AMGHALIA M. S. ELMNAFI

Date: 2 November 2009
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICACATION</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

### CHAPTER

#### I INTRODUCTION

1

#### II LITERATURE REVIEW

*Staphylococcus aureus*

- Infections caused by *Staphylococcus aureus* 8
- Syndromes caused by *Staphylococcus aureus* 9
- Biological properties of *Staphylococcus aureus* 10
- Cell wall 11

*Staphylococcus aureus* colonization, virulence factors and defense Mechanism 12

*Staphylococcus aureus* Infections in Malaysia 14

- Epidemiology of *Staphylococcus aureus* 16
- Identification of *Staphylococcus aureus* 16
- Gram Stain 17
- Conventional biochemical tests 17
- Multitest System biochemical test 19

- Rapid identification of *Staphylococcus aureus* 20
- Current issues on *Staphylococcus aureus* 21
- Antibiotic resistance in *Staphylococcus aureus* 23
- Methicillin sensitive *Staphylococcus aureus* (MSSA) 24
- Methicillin resistant *Staphylococcus aureus* (MRSA) 24

- Genetic polymorphism 26
- Molecular analysis 29
- Polymerase Chain Reaction (PCR) 34
- Types of PCR 37
- Randomly Amplified Polymorphic DNA 38
- Repetitive Enteric Polymerase Chain Reaction 43
III MATERIALS AND METHODS

Source of bacterial isolates
Bacteriological characterization tests
Growth on blood agar selective medium
Gram staining
Catalase activity
Coagulase test
Oxidase activity
Preparation of stock culture of bacteria
Antimicrobial susceptibility testing
DNA extraction
Total DNA extraction
Quantity and Purity of DNA
Agarose gel electrophoresis of DNA
RAPD-PCR assay protocol
RAPD fingerprinting
RAPD analysis
Repetitive Element Sequence based Polymerase Chain Reaction (rep-PCR) DNA fingerprinting
Fragment Size, RAPD and Rep-PCR Band Scoring
16S rRNA amplification
Restriction enzyme digestion
Sequencing of 16S rRNA
Detection of mecA, mecR1 and mecI genes
DNA sequencing of mecA, mecR1 and mecI genes
Amplification of adaB gene
DNA sequencing of adaB gene

IV RESULTS

Bacterial source
Characterization tests
Growth on blood agar plate
Gram staining and biochemical tests
Antibiotic susceptibility test
Total genomic DNA extraction
DNA quantitation
RAPD and rep-PCR Banding Profile
Computer Analysis of RAPD and rep-PCR Fingerprints
Genetic Distances obtained by RAPD –PCR Fingerprint Analysis
Genetic Distances obtained by Rep–PCR Fingerprint Analysis
Genetic Markers obtained from RAPD and Rep Fingerprints
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical <em>Staphylococcus aureus</em> isolates used in this study</td>
<td>51</td>
</tr>
<tr>
<td>Clinical <em>Staphylococcus aureus</em> isolates used RAPD and Rep-PCR fingerprinting</td>
<td>59</td>
</tr>
<tr>
<td>Primers screened for random amplification (kit AE)</td>
<td>61</td>
</tr>
<tr>
<td>Primers used in rep-PCR</td>
<td>64</td>
</tr>
<tr>
<td>Primers used for MRSA genes amplification</td>
<td>68</td>
</tr>
<tr>
<td>The sensitivity of <em>S. aureus</em> isolates to different antibiotics</td>
<td>72</td>
</tr>
<tr>
<td>The DNA purity and concentration obtained for <em>S. aureus</em> isolates studied</td>
<td>77</td>
</tr>
<tr>
<td>Mutations detected in 16S rRNA gene of clinical <em>S. aureus</em> isolates</td>
<td>100</td>
</tr>
<tr>
<td>Mutations detected in <em>mecA, mecRI</em> and <em>mecI</em> genes of MRSA isolates</td>
<td>106</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Golden yellowish, creamy and opaque colonies of <em>S. aureus</em> on the blood agar plate</td>
<td>70</td>
</tr>
<tr>
<td>The sensitivity of <em>S. aureus</em> isolates to different antibiotics</td>
<td>72</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>75</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>75</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>75</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>76</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>76</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>76</td>
</tr>
<tr>
<td>Genomic DNA extracted from <em>S. aureus</em> isolates</td>
<td>77</td>
</tr>
<tr>
<td>RAPD obtained with primer OPAE-06</td>
<td>81</td>
</tr>
<tr>
<td>RAPD obtained with primer OPAE-10</td>
<td>82</td>
</tr>
<tr>
<td>RAPD obtained with primer OPAE-14</td>
<td>83</td>
</tr>
<tr>
<td>RAPD obtained with primer OPAE-15</td>
<td>84</td>
</tr>
<tr>
<td>REP genetic profile obtained with primer REP1</td>
<td>85</td>
</tr>
<tr>
<td>REP genetic profile obtained with primer REP2</td>
<td>86</td>
</tr>
<tr>
<td>REP genetic profile obtained with primer REP3</td>
<td>87</td>
</tr>
<tr>
<td>RAPD Dendrogram of genetic relationship between 50 <em>S. aureus</em> isolates</td>
<td>88</td>
</tr>
<tr>
<td>REP Dendrogram of genetic relationship between 50 <em>S. aureus</em> isolates</td>
<td>93</td>
</tr>
<tr>
<td>Electrophoresis of PCR amplification using universal primer on clinical isolates</td>
<td>95</td>
</tr>
<tr>
<td>Electrophoresis of banding profile of amplified products digested with <em>HaeIII</em></td>
<td>96</td>
</tr>
</tbody>
</table>
Sequencing results of 16S rRNA of clinical isolates 100

Dendrogram of clinical *S. aureus* isolates clustering based on 16S rRNA sequence 101

The detection of *mecA* gene fragments by PCR 102

The detection of *mecR1* gene fragments by PCR 102

The detection of *mecI* gene fragments by PCR 103

Sequence of *mecA* gene in *S. aureus* 104

Sequence of *mecR1* gene in *S. aureus* 105

Sequence of *mecI* gene in *S. aureus* 106

Electrophoresis of *adaB* gene from the PCR products 107
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-PCR</td>
<td>arbitrary primed polymerase chain reaction</td>
</tr>
<tr>
<td>BA</td>
<td>blood agar</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EB</td>
<td>elution buffer</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HTAA</td>
<td>Hospital Tunku Ampuan Afzan</td>
</tr>
<tr>
<td>HUSM</td>
<td>Hospital Universiti Sains Malaysia</td>
</tr>
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<td>kb</td>
<td>kilo base Pair</td>
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<td>l</td>
<td>litre</td>
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<td>LB</td>
<td>Luria Bertanii</td>
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<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>milliliter</td>
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<td>MgCl</td>
<td>magnesium chloride</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>Ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
<tr>
<td>UMMC</td>
<td>Universiti Malaya Medical Center</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Staphylococci are often found in the human nasal cavity (and on other mucous membranes) as well as on the skin. There are five species of staphylococci commonly associated with clinical infections: *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. saprophyticus*.

*S. aureus* is the most pathogenic species and is implicated in a variety of infections. The bacterium causes a variety of suppurative (pus-forming) infections and toxinoses in humans. The increasing prevalence of antibiotic resistant *S. aureus* has become an additional problem (Van Leeuwen *et al.*, 1999). Over the past 2 decades; antimicrobials have become increasingly available for a broad range of pathogens. Due to the widespread and uncontrolled use of these drugs, new forms of antimicrobial resistance have emerged. Worldwide, *S. aureus* have been identified as resistant to erythromycin, fusidic acid and lately even to the last antibiotic of choice, the vancomycin. *S. aureus* strains resistant to methicillin, the 3rd generation antibiotic before vancomycin have been reported shortly after the introduction of penicillinase-resistant β-lactams in 1960.
Methicillin-resistant *Staphylococcus aureus* or “MRSA” are staph bacteria that have become resistant to beta-lactam antibiotics, including: penicillin, ampicillin, amoxicillin, amoxicillin/clavulanate, methicillin, oxacillin, dicloxacillin, cephalosporins, carbapenems (e.g., imipenem), and the monobactams (e.g., aztreonam). MRSA causes the same types of infections as staph bacteria that are sensitive to beta-lactam antibiotics. MRSA worldwide have become an important cause of nosocomial infections (Doebbling, 1995) and the infection burden in healthcare facility is a global scenario and remained a central issue in multiple drug resistant infection management. The disease burden reported in 2006 for United States afflicted an estimated 2 million patients in each year, which represents up to 5% of hospitalized patients and results in an estimated 88,000 deaths and 4.5 billion dollars in excess health care costs. Understanding MRSA pathogen relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. A valuable consequence of advances in molecular biology is the applicability of molecular approach for determining molecular relatedness of isolates for epidemiologic investigation through new technologies based on DNA, or molecular analysis. These DNA-based molecular methodologies include PCR-based typing methods of genomic DNA or relevant target genes such as virulent factors or antibiotic determinants. Establishing clonality of pathogens can aid in the identification of the source (environmental or personnel) of organisms, distinguish infectious from noninfectious strains, and distinguish relapse from reinfection. The integration of molecular typing with conventional hospital epidemiologic surveillance has been proven to be cost-effective due to the associated reduction in the number of nosocomial infections (Singh *et al.*, 2006). Cost-effectiveness
is maximized through the collaboration of the laboratory, through epidemiologic typing, and the infection control department during epidemiologic investigations.

The implementation of molecular approach requires the establishment and optimization of molecular protocols in any diagnostic laboratories which include microbiology laboratories that conduct bacteriology investigation such as identification and characterizations. Establishment of databases on molecular properties of local strains is a sound approach in an epidemiological-linked health infection management.

Among molecular properties of pathogens that are useful in an epidemiology based management include sequence analysis of species specific target genes, universal 16S rRNA genes, antibiotic resistant determinant genes, relevant infective site genes or virulent factor genes as well as genetic diversity and clonal types. Other important molecular traits useful in infection management are colonization versus infection gene markers as well as source tracing gene markers. These properties can contribute immensely to surveillance program to reduce and control infection from multiple drug resistant pathogens.

Identification of the causative agent of any disease as early as possible is very important to begin appropriate antibiotic treatment, in order to curtail serious infection. Bacteria get multiplied when ingested into the body in a very short time and some bacteria
produce toxin that lead to severe illness in human or animals. Identification of bacteria is fundamental to bacteriology in general and is crucial in applied bacteriology. Eventual classification of the organism is important, not only for epidemiological purposes but also for verification of the pathogen identity. Throughout the years, several methods for identifying *S. aureus* have been evaluated. Methods for the identification of *S. aureus* include conventional methods which include culture method, Gram stain, catalase tests, coagulase tests and immunological tests such as antigen and antibody tests and a panel of commercial agglutination testes, hybridization test for rRNA, an enzymatic test for the detection of thermo stable nuclease and the molecular method.

The culture method is the most sensitive one among the various methods currently used in clinical laboratory for detection of bacterial infections. However, culture requires at least 8 hours of incubation and additional time is needed to perform biochemical or immunological tests to identify the bacteria (Lu *et al.*, 2000). In addition the detection of resistance by conventional methods may not always be easy or possible. Conventional antimicrobial susceptibility testing methods require that pathogens are first isolated from human specimens by culture methods. In separate assays, isolated microorganisms are then exposed to various concentrations of antimicrobial agents under specified growth conditions, and the ability of these antimicrobics to inhibit growth is determined. Methods that are frequently used for testing cultivated bacteria and yeasts include disk diffusion, broth dilution, agar dilution, and gradient diffusion (Epsilometer test).

Other methods such as antibody and antigen detection may suffer from false negative reactions, cross-reactions, background titers, and non-specificity. Although identification
with the thermo nuclease enzyme test has shown an excellent correlation with the other conventional identification methods, false-positive results due to thermo nuclease activity in some strains of coagulase-negative *Staphylococcus* may occur. Excellent specificity but variable sensitivity was noted with diagnostic kits based on agglutination for identification directly from clinical specimens. Furthermore, several of these kits, which are based on agglutination, fail to detect methicillin-resistant staphylococci. The hybridization tests for rRNA showed excellent specificity for *S. aureus* but evidently demonstrate a lack of sensitivity for detection from blood cultures (Pitt *et al.*, 2000).

In order to get more reliable results at a shorter time with a less cost, many researchers are studying bacteria using different approaches. Accurate and rapid epidemiologic typing is crucial for the identification of the source and spread of infectious disease. The epidemiology of *S. aureus* infections needs to be studied, for this can be done by the application of multiple typing techniques based on the detection of DNA polymorphisms have been developed and optimized. A variety of typing techniques is available to help determine the source and transmission routes of *S. aureus* strains (Tambic *et al.*, 1997). One of the genotypic methods used in epidemiological studies of *S. aureus* is PCR-based methods.

Once the causative organism for any infection or disease is identified, the appropriate treatment can commence in which it is important to choose the suitable antibiotic. As *S. aureus* is representative of multiple antibiotic resistant bacteria, it is necessary to study the ways to control this bacterium and their ability to counteract antibiotic effects. The
investigation or molecular properties of the drug resistant could lead to improved treatment method through discovery of new antimicrobial agent or new drug target molecular sites of the resistant pathogen. Worldwide, many strains of \textit{S. aureus} are already resistant to all antibiotics and thus the organism has progressed one step closer to becoming an unstoppable killer. To efficiently prevent dissemination of these pathogens, rapid and reliable identification procedure for \textit{S. aureus} by the amplification of multiple resistant antibiotic gene determinants by PCR in order to efficiently support therapy and eradication of the pathogen is needed.

In the wake of urgent need to establish effective infection control strategies for MRSA in health care settings through enhanced surveillance, strategies focusing on early and accurate detection through strain identity, antibiotic susceptibility gene determinants as well as genetic diversity of strains prompted the establishment of the general objectives of the research which is to assess some properties of local MRSA isolates by utilizing the molecular tools. The objective will be achieved by performing the investigations stipulated in the specific objectives which include:

1- To determine and compare DNA fingerprint pattern of MRSA and non-MRSA by using an arbitrary primers.

2- To determine clonal differences of MRSA with non-MRSA isolates.

3- To evaluate 16S rRNA sequences of MRSA and non-MRSA.

4- To evaluate variation in MRSA isolates based on mecA, mecI and mecR1 gene sequences
Undertaking the increased awareness and appropriate management is universally indicated. A need to emphasize monitoring patient management and establishing the local prevalence of antimicrobial resistance in specific geographical locations, according to growth remained beneficial. However, due to the lack of data on molecular typing to guide management, it is imperative that such data be established for local bacterial strains. Multiple DNA-based methods have been introduced to genetically type *S. aureus* strains, but not a single technique appeared to be universally applicable. Most of the current image-based approaches generate complex banding patterns and lack generally accepted interpretation criteria. The need for straightforward and reproducible techniques generating simple output that can be used for computerized data-management still is an important research topic.
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151


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antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochemica et Biophysica Acta* 73: 57-70.


