UNIVERSITI PUTRA MALAYSIA

MOLECULAR EPIDEMIOLOGY CHARACTERIZATION OF CLINICALLY IMPORTANT STAPHYLOCOCCUS AUREUS

AMGHALIA M. S. ELMNAFI

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MOLECULAR EPIDEMIOLOGY CHARACTERIZATION OF CLINICALLY IMPORTANT *STAPHYLOCOCCUS AUREUS*

By

AMGHALIA M. S. ELMNAFI

**Thesis Submitted to the School of Graduate Studies,
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Degree of Master of Science**

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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

Faculty : Medicine and Health Sciences

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

AMGHALIA M. S. ELMNAFI

November 2009

Pengerusi : Mariana Nor Shamsudin, PhD

Fakulti : Perubatan dan Sains Kesihatan

Staphylococcus aureus tahan metisilin (MRSA), sejenis jangkitan yang diperolehi di hospital telah menjadi penyebab bagi jangkitan-jangkitan kulit serta invasif di kalangan orang dewasa mahupun kanak-kanak sihat dalam komuniti. Penyelidikan ini telah berjaya menetapkan sifat-sifat molekular isolat-isolat klinikal MRSA untuk membezakannya daripada isolat-isolat *Staphylococcus aureus* peka metisilin (MSSA). Sifat-sifat molekular MRSA tempatan yang berkenaan menyumbang sebagai pengkalan data permulaan bagi isolat-isolat ini yang bertujuan memahami epidemiologi, mikrobiologi dan patofisiologi jangkitan-jangkitan ini dengan sepenuhnya. Kajian ini mengandungi sejumlah 90 isolat-isolat dari lokasi-lokasi berlainan di Malaysia.

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 pemprofilan 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.

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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.

The members of the Supervisory Committee were as follows:

Mariana Nor Shamsudin, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairperson)

Raha Abdul Rahim, Ph.D.
Professor
Faculty of Food Sciences and Biotechnology
Universiti Putra Malaysia
(Member)

Zamberi Sekawi
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
University Putra Malaysia

Data: 11 February 2010

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LIST OF ABBREVIATIONS

AP-PCR	-arbitrary primed polymerase chain reaction
BA	-blood agar
bp	-base pair
°C	-degrees centigrade
DNA	-deoxyribonucleic acid
dNTPs	-deoxynucleotide triphosphate
EB	-elution buffer
EDTA	-ethylenediamine tetraacetatic acid
g	-gram
HTAA	-Hospital Tunku Ampuan Afzan
HUSM	-Hospital Universiti Sains Malaysia
kb	-kilo base Pair
l	-litre
LB	-Luria Bertanii
mg	-milligram
ml	-milliliter
MgCl	-magnesium chloride
MIC	-minimum inhibitory concentration
MRSA	-methicillin resistant *Staphylococcus aureus*
MSSA	-methicillin sensitive *Staphylococcus aureus*
NCCLS	-National Committee for Clinical Laboratory Standards
Ng	-nanogram

PBP	-penicillin binding protein
PCR	-polymerase chain reaction
rDNA	-Ribosomal DNA
RAPD	-Random amplified polymorphic DNA
TBE	-tris-borate-EDTA
UMMC	-Universiti Malaya Medical Center
UV	-ultra violet
V	-volt
µg	-microgram
µl	-microlitre

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 b -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 rRNAgenes, 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 *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 *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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