UNIVERSITI PUTRA MALAYSIA

PHENOTYPE AND GENOTYPE CHARACTERISATION OF MACROLIDE, LINCOSAMIDE AND STREPTOGRAMIN B RESISTANCE AMONG HOSPITAL AND COMMUNITY ISOLATED Staphylococcus aureus STRAINS

ASMA MESBAH EL.KAMMOSHI

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By

ASMA MESBAH EL.KAMMOSHI

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

February 2016
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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By

ASMA MESBAH ELKAMMOSHI

February 2016

Chair : Assoc. Prof. Rukman Awang Hamat, PhD
Faculty : Medicine and Health Science

\textit{Staphylococcus aureus} is an important cause of nosocomial and community-acquired infections in every region of the world. The success of \textit{S. aureus} as a pathogen and its ability to cause such a wide range of infections is due to its extensive virulence factors. The increase in the resistance of this virulent pathogen to antibacterial agents, coupled with its increasing prevalence as a nosocomial pathogen, is a cause for consternation. The escalating frequency of \textit{S. aureus} infections and the changing patterns in antimicrobial resistance have led to renewed interest in the use of macrolide lincosamide–streptogramin B (MLSb) antibiotics to treat such infections. Therapeutic failure to clindamycin has been reported due to mechanisms which confer resistance constitutively, or by the presence of low level inducers. Clindamycin is one of the alternative agents used to treat \textit{S. aureus} infections and accurate identification of clindamycin resistance is important to prevent therapeutic failure. Unfortunately, inducible clindamycin resistance is not detected by standard susceptibility tests. Also, the incidence of drug-resistant pathogens differs greatly between countries according to differences in the usage of antibiotics. This research was carried out in order to study the prevalence of iMLSb in community associated (CA) and hospital associated (HA) \textit{S. aureus} isolates from clinical samples, and for the presence of macrolide and lincosamides resistance genes [\textit{erm(A)}, \textit{erm(B)}, \textit{erm(C)} and \textit{msr(A)}].

A total of 133 and 50 isolates of clinical and community acquired samples, respectively were obtained from various sites. Disk diffusion testing by placing clindamycin and erythromycin disks 15 mm and 26 mm apart (edge to edge) on a Mueller-Hinton agar, as per CLSI guideline and E-test methods were performed. The result showed that only four out of 183 (2.2\%) clinical isolates were resistant to erythromycin. Of these four isolates, one (25\%) showed MS phenotype (erythromycin resistance; clindamycin susceptible) with \textit{msr(A)} gene detected and the remaining three (75\%) isolates exhibited D-phenotype (erythromycin resistance; clindamycin resistance) and positive for \textit{erm(C)} gene. Similar findings were observed regardless of two different distances used for the screening of MLSb
phenotypes. In addition, all 50 community isolates were sensitive to erythromycin and clindamycin. However, the isolates that showed MS phenotype and D-positive had different spa-types which show a diverse genetic heterogeneity.

In spite of the low prevalence of *S. aureus* with iMLS\(\text{b}\), it is quite interesting and significant to find that they were mostly isolated from inpatients. D-test becomes an imperative part of routine antimicrobial susceptibility test for all *S. aureus* isolates. Inducible clindamycin resistance testing should be done as a routine practice. Failure to inculcate this practice can lead to ineffective treatment options and ultimately irrational use of other higher class of antibiotics.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN FENOTIP DAN GENOTIP KERENTANAN MAKROLID, LINKOSAMID DAN STREPTOGRAMIN B DI KALANGAN HOSPITAL DAN KOMUNITI STRAINS Staphylococcus aureus YANG DIPENCILKAN

Oleh

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Staphylococcus aureus adalah punca utama jangkitan nosokomial dan perolehan kmuniti di setiap rantau di dunia. Kejayaan S. aureus sebagai patogen dan keupayaan untuk menyebabkan pelbagai jenis jangkitan adalah disebabkan oleh faktor virulens yang luas. Peningkatan rintangan patogen ini virulen kepada agen anti-bakteria, ditambah pula dengan kelaziman yang semakin meningkat sebagai patogen nosokomial, adalah suatu perkara yang membingungkan. Kekerapan peningkatan jangkitan S. aureus dan perubahan corak dalam rintangan antimikrob telah membawa kepada minat dalam penggunaan makrolid linkosamid-streptogramin B (MLSb) antibiotik untuk merawat jangkitan tersebut. Kegagalan terapi untuk klindamisin telah dilaporkan disebabkan oleh mekanisme-mekanisme yang memberikan rintangan konstitutif, atau dengan kehadiran pencetus tahap rendah. Klindamisin adalah salah satu agen alternatif yang digunakan untuk merawat jangkitan S. aureus dan pilihan yang tepat terhadap rintangan klindamisin adalah penting untuk mencegah kegagalan terapeutik. Malangnya, rintangan klindamisin tercetus tidak dapat dikecualkan oleh ujian kecenderungan standard. Juga, kejadian ubat tahan patogen sangat berbeza antara negara-negara mengikut perbezaan dalam penggunaan antibiotik. Kajian ini telah dijalankan dalam usaha untuk mengkaji kelaziman iMLSb dalam isolate perolehan komunti (CA) dan perolehan hospital (HA) S. aureus daripada sampel klinikal, dan kehadiran gen-gen makrolid dan linkosamid rintangan [erm(A), erm(B), erm(C) dan msr(A)].

Sebanyak 133 dan 50 isolat sampel klinikal dan komuniti yang diperolehi, masing-masing telah diperoleh dari pelbagai tapak. Ujian resapan cakera dengan meletakkan klindamisin dan eritromisin dalam jarak 15 mm dan 26 mm (sisi ke sisi) pada agar Mueller-Hinton seperti di dalam garis panduan CLSI dan kaedah E-test telah dijalankan. Hasil kajian mendapati hanya empat daripada 183 (2.2%) isolat klinikal adalah tahan kepada eritromisin. Daripada empat isolat, satu (25%) menunjukkan fenotip MS dengan msr(A) gen dikesan dan baki tiga (75%) mempamerkan D-fenotip dan positif untuk erm(C) gen. Penemuan yang serupa diperhatikan untuk dua jarak yang digunakan untuk saringan fenotip MLSb. Di samping itu, semua 50 isolat
komuniti adalah sensitif kepada eritromisin dan klindamisin. Walau bagaimanapun, isolat yang menunjukkan MS fenotip dan D-positif mempunyai spa yang berbeza-jenis yang menunjukkan kepelbagaian genetik yang pelbagai.

Walaupun kelaziman yang rendah di antara \textit{S. aureus} dengan iMLS\textsubscript{b}, ia agak menarik dan penting untuk mendapati bahawa mereka kebanyakannya diasingkan daripada pesakit dalaman. Ujian D menjadi bahagian yang penting daripada ujian rutin kecenderungan antimikrob untuk semua \textit{S. aureus} yang diasingkan. Ujian rintangan klindamisin perlu dilakukan sebagai amalan rutin. Kegagalan untuk memupuk amalan ini boleh membawa kepada pilihan rawatan tidak berkesan dan penggunaan akhirnya terarah kepada pilihan antibiotik kelas tinggi.
I would never have been able to finish my dissertation without the guidance of my committee members, help from friends, and support from my family and husband.

I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr Rukman Awang Hamat for his excellent guidance, caring, patience, generous contribution of knowledge and experience, valuable comments and encouragement from the start until the end of my study and providing me with an excellent atmosphere for doing research. Additionally, I would like to thank my committee members Dr. Suresh Kumar Subbiah for his interest in my work. I was really privileged to have him as my co-supervisor. I am grateful to the members of Medical Microbiology and Parasitology Department at University Putra Malaysia for their comradeship. I would like to express special thanks to Medical Microbiology student members for being helpful in this research. Finally, I must express my very profound gratitude to my parents and to my husband for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.
I certify that a Thesis Examination Committee has met on 3 February 2016 to conduct the final examination of Asma Mesbah El. Kammoshi on her thesis entitled "Phenotype and Genotype Characterisation of Macrolide, Lincosamide and Streptogramin B Resistance among Hospital and Community Isolated Staphylococcus aureus Strains" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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School of Graduate Studies  
Universiti Putra Malaysia  

Date:
Declaration by graduate student

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This is to confirm that:
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- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate studies) Rules 2003 (Revision 2012-2013) are adhered to.

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Name of Chairman of Supervisory Committee: Rukman Awang Hamat, PhD

Signature: __________________________
Name of Member of Supervisory Committee: Suresh Kumar Subbiah, PhD
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>CA-MSSA</td>
<td>Community-associated methicillin-sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>HA-MSSA</td>
<td>Hospital-associated methicillin-sensitive <em>Staphylococcus aureus</em></td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleotide</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<tr>
<td>spa</td>
<td>Staphylococcal protein A</td>
</tr>
<tr>
<td>erm</td>
<td>Erythromycin ribosomal methylase</td>
</tr>
<tr>
<td>msr</td>
<td>Macrolide-streptogramin resistance</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
</tr>
<tr>
<td>SAB</td>
<td>Staphylococcus aureus bacteremia</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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CHAPTER 1

INTRODUCTION

1.1 Background

*Staphylococcus aureus* is a Gram positive bacterium which was first recognized in the 1880s as a potential pathogen, primarily responsible for the colonization of multiple tissues and organs in both humans and animals (Wertheim, Melles, Vos, Van, Verbrugh & Nouwen 2005). *S. aureus* is part of normal flora of the human body, skin and nasopharynx and at every point in time, about 20-30% of the human population get colonized by *S. aureus* (Harris, Foster & Richards, 2002). *S. aureus* is thus regarded as one of serious opportunistic pathogens that causes a variety of infections such as mild skin and soft tissue infections, bacteremia complicated by endocarditis, pneumonia, metastatic infections, sepsis or toxic shock syndrome in both immunocompromised patients and immunocompetent individuals (Harris et al., 2002).

The use of antibiotics such as methicillin was the main option available for treating *S. aureus* infections in the 1940s but unfortunately, the euphoria that welcomed the introduction of penicillin turned into grave consternation barely two years after it was introduced when some strains of *S. aureus* begun exhibiting resistance to penicillin. Resistance to this “miracle drug” was first recognized in strains of *S. aureus* which produce penicillinase and then all hope seemed lost when by 1950, the rate at which resistance occurred mandated the use of alternative therapy (Otto, 2010). Strains of *S. aureus* with an altered penicillin-binding protein soon countered the semi-synthetic beta-lactam antibiotics that had replaced penicillin (Chambers & Deleo, 2010). For many years, MRSA was mainly a hospital-associated infection but in the 21st century, MRSA evolved beyond the “hospital walls” to become an exceptional “Community-Associated” public health challenge. The rate at which staphylococcal infections are resistant to antimicrobials is rapidly increasing. In actual fact, *staphylococcus* is the most widely isolated organism among inpatients in Malaysian hospitals. The rate of isolation is between 1.6 and 5.5 for every 100 patients. Of the *S. aureus* isolated, 0.2 to 2.3% is usually resistant to methicillin and this is commonly isolated from surgical, pediatrics, orthopedics and intensive care units (Koh, Husni, Tan, Kunaseelan, Nuriah & Morad, 2009).

*S. aureus* infections are globally being reported as an important cause of hospital associated (HA) and community associated (CA) infections (David & Daum, 2010). Staphylococcal infections which mostly affect the skin and soft tissues are reported to involve nearly a third of the global population and in countries like the USA where MRSA is endemic, over ten million outpatient visits and nearly a half-million hospital admissions per year is accountable to *S. aureus* infection (Gorwitz, Kruszon, McQuillan, McDougal, Fosheim & Kuehnert, 2008; Klein, Smith & Laxminarayan, 2007; McCaig, McDonald, Mandal & Jemigan, 2006). Reports show that MRSA infections kill about 19,000 hospitalized American patients every year and this figure is similar to that of a report on the total deaths caused by combination of HIV/AIDS, tuberculosis and viral hepatitis (Klevens, Morrison, Nadle, Petit & Gershman, 2007;
Boucher & Corey, 2008). Apart from the fact that *S. aureus* infection leads to significant morbidity and mortality compared to infections prompted by other pathogens, there is also a disproportionate burden on the financial state of affected societies especially their health care resources (Wisplinghoff, Bischoff, Tallent, Seifert, Wenzel & Edmond, 2004).

Due to the heightened resistance to antimicrobial agents among Staphylococci, other antibiotics which had good pharmacokinetic properties were sought and clindamycin which is a Lincosamides, became the choice antibiotic used in combatting *S. aureus* infections (Lyall, Gupta & China, 2013). Since its discovery, clindamycin has been an effective preferred treatment option for both methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) infections especially with penicillin allergic patients (Patel, Waites, Moser, Cloud & Hooley, 2006). However, their continuous and extensive use has led to a notable resistance among staphylococcal strains thus leading to difficulty in treating infections caused by these microbial agents (Noskin, Rubin, Schentag, Kluytmans, Hedblom, Jacobson & Bharmal, 2007). Whilst macrolides (erythromycin, dirithromycin, clarithromycin, azithromycin), lincosamides (clindamycin and lincomycin), and streptogramin B (quinupristin/dalfopristin) belong to different classes of antimicrobials, they all act by the inhibition of protein synthesis. Thus, D-test screening could be used to determine the resistance types of these antibiotics in diagnostic laboratories.

The resistance exhibited by staphylococcal isolate towards the MLSβ group of antibiotics is mainly effectuated by multiple mechanisms such as target site modification, active efflux, and enzymatic drug inactivation. Erythromycin ribosomal genes (*erm*) are known to be responsible for the alteration of the antibiotic target site. It is believed that changes in A2058 nucleotide would lead to the impairment of the antibiotic binding to the target site. A specific efflux pump is encoded by the *msr*(A) gene. This energy dependent pump effectively expels macrolides and streptogramin B from bacterial cell before they bind to their binding site on the ribosome. Notably, this mechanism of resistance does not create resistance to clindamycin (MS phenotype), which is active against such isolates. Inactivation of MLSβ antibiotics confers resistance to structurally related antibiotics only. Esterases, phosphotransferases, acetyltransferases, hydrolases, and nucleotidyltransferases have been identified in strains that are resistant to macrolides or lincosamides (Leclercq, 2002).

It is of utmost necessity for laboratories to draw a clear line between MS (clindamycin sensitive) and iMLSβ (clindamycin resistance) resistance before reporting an erythromycin-non susceptible *Staphylococcus* sp. isolate as clindamycin susceptible. This differentiation can be done by the erythromycin-clindamycin disk approximation test or D-test. When an organism expressing iMLSβ resistance, it is tested according to Clinical and Laboratory Standards Institute (CLSI) methods with a 15-μg erythromycin disk placed between 15-26mm close to a 2μg clindamycin disk, the zone of inhibition around the clindamycin disk is flattened to form a “D” shape (positive D-test). Whereas in the MS phenotype, the clindamycin zone remains circular (sensitive) while it is resistant to erythromycin. A false-negative D-test will result in reporting an isolate as clindamycin susceptible when it should be reported as resistant (very major error), while a false-positive test will result in reporting an
isolate as resistant when it should be reported as susceptible (major error) (O’Sullivan, Cai, Kong, Zeng & Gilbert, 2006).

In order to make accurate decision regarding therapy, the importance of authentic susceptibility data cannot be over-emphasized. However, only a few published articles are available about the prevalence of erythromycin and clindamycin resistance in Malaysian isolates of *S. aureus*. Also, false susceptibility results for clindamycin may be obtained if isolates are not tested for iMLSв resistance. This type of resistance cannot be determined by using standard susceptibility test alone thus and need the use of D-test is recommended (Fiebelkorn, Crawford, McElmeel & Jorgensen, 2003).

The *spa* typing technique uses the sequence of a polymorphic VNTR in the 3’ coding region of the *S. aureus*-specific staphylococcal protein A (*spa*). Each new base composition of the polymorphic repeat found in a strain is assigned a unique repeat code. The repeat succession for a given strain determines its *spa* type. The individual repeat length for the *spa* VNTR is usually 24bp, but exceptions of 21 to 30 do exist. Although, *spa* typing is a single-locus typing technique, it offers a subtyping resolution comparable to more expensive and/or laborious techniques such as MLST and PFGE. The technique is widely used for sub-typing of *S. aureus* in hospital and outbreak settings. In addition, based on previous studies there is no specific *spa* type of *S. aureus* showing any relationship with clindamycin resistance (Wang, Chiueh, Sun, Tsao & Lu, 2012; Uzunović, Ibrahimagić, Kamberović, Kunarač, Rijnders & Stobberingh, 2013).

### 1.2 Problem Statement

Globally, bacterial infections keep on causing significant morbidity and mortality among affected individuals. The resistance to antimicrobial agents among staphylococci is an alarming problem. This has led to renewed interest in the usage of MLSв antibiotics to treat *S. aureus* infections. Clinical failure of clindamycin therapy has been reported due to the resistance to MLSв antibiotics. *In vitro* routine tests for clindamycin susceptibility may fail to detect inducible clindamycin resistance due to the presence of *erm* genes resulting in treatment failure, thus necessitating the need to detect such resistance by a simple D-test as a routine basis (Yılmaz, Iskender, Caylan & Koksal, 2007).

### 1.3 Significance of Study

The inaccuracy of detecting inducible clindamycin resistance stands as a serious challenge because there will be a failure to give appropriate treatment since clindamycin sensitivity is erroneously reported by the lab. This study was carried out in order to study the characteristics of macrolides, lincosamides and streptogramin B (MLSв) resistance in both hospital and community-acquired methicillin-sensitive *Staphylococcus aureus* (MSSA) using a clindamycin-erythromycin disk approximation test, and to detect the presence of resistance genes among these isolates.
1.4 Objectives:

1.4.1 General objective

The main aim of this research was to study the phenotypic and genotypic characteristics of macrolides and lincosamides antibiotics resistance in MSSA among clinical isolates.

1.4.2 Specific objectives

1. To screen the phenotypic MLSβ characteristics (iMLSβ, cMLSβ, MS) of MSSA isolates by D-test.
2. To detect the resistant genes \([erm(A), erm(B) and erm(C)]\) for iMLSβ, cMLSβ phenotypes, and \(msr(A)\) for MS phenotype.
3. To associate the specific \(spa\) type that is related to clindamycin resistance.
REFERENCES


Cetin, E. S., Gunes, H., Kaya, S., Aridogan, B. C., & Demirci, M. (2010). Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among clinical staphylococcal isolates in a Turkish university hospital. *Journal of Microbiology, Immunology and Infection*, 43(6), 524-529


Determined by Multiplex PCR Assays and the Antibiotic Susceptibility Patterns of Staphylococcus aureus and Staphylococcus epidermidis. Antimicrobial agents and chemotherapy, 44(2), 231-238.


## APPENDIX

### Preparation of Media, Buffers and Reagents

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE buffer</strong></td>
<td>a. 10Mm Tris-HCL, pH 8.0 (1.31g Tris-HCL in 200ml of distilled water at room temperature and adjusted to pH to 8.0)</td>
</tr>
<tr>
<td></td>
<td>b. 1mM Na-EDTA, pH 8.0 (add 0.05g Na-EDTA in 200ml distilled water and adjusted to the pH to 8.0)</td>
</tr>
<tr>
<td></td>
<td>c. The buffer was autoclaved at 121°C for 15 min and stored at room temperature</td>
</tr>
<tr>
<td><strong>TBE buffer</strong></td>
<td>108 g/l Tris-base (C14H11NO3) 55 g/L Boric acid (H3BO3), 9.13 g/L Ethylenediamine tetraacetic acid. The above chemicals were dissolved in distilled water and the pH was adjusted to 8.3 adding NaOH until dissolved, then topping up with distilled water to a final volume of 1000ml. The buffer was autoclaved and stored at room temperature.</td>
</tr>
<tr>
<td><strong>Lysozyme stock solution</strong></td>
<td>0.5mg of lysozyme powder (Sigma, Germany) was dissolved into 1ml of distilled water (0.5mg/ml). It was sterilized by using 0.5μm filter paper and stored at room temperature.</td>
</tr>
<tr>
<td><strong>0.85% Nacl</strong></td>
<td>0.125g of Nacl was measured and dissolved in 100ml of distilled water. The prepared saline was autoclaved and stored at room temperature</td>
</tr>
<tr>
<td><strong>70% ethanol</strong></td>
<td>70 ml of absolute ethanol was measured into a dispensing container. 30 ml of distilled water was added to make up 100ml of 70% ethanol</td>
</tr>
<tr>
<td><strong>Muller-hinton agar</strong></td>
<td>34g of powdered media is suspended as the manufacture recommendations in 1L distilled water. Suspension is boiled to dissolve the medium completely. The dissolved media is sterilized by autoclaving at 121°C for 15minutes. The media is leaved to cool and poured into sterile plates.</td>
</tr>
</tbody>
</table>