



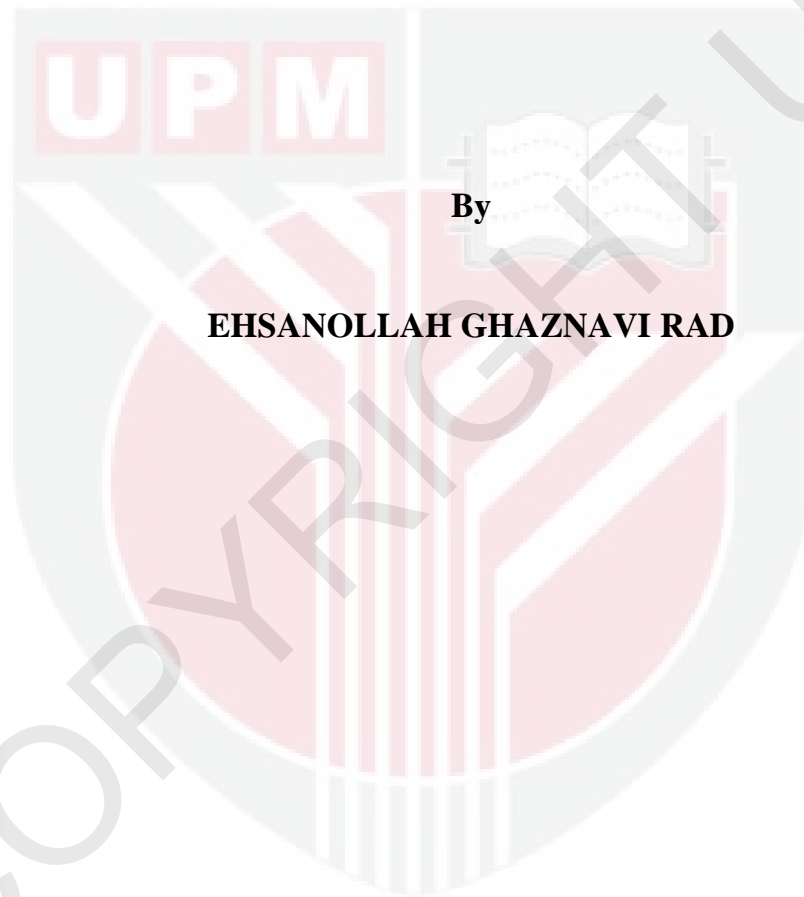
UNIVERSITI PUTRA MALAYSIA

**MOLECULAR EPIDMIOLOGY AND THERAPEUTIC POTENTIAL OF
PERSIAN SHALLOT (*Allium ascalonicum* L.) IN THE MANAGEMENT OF
METHICILLIN RESISTANT *Staphylococcus aureus* INFECTION**

EHSANOLLAH GHAZNAVI RAD

FPSK(p) 2010 3

**MOLECULAR EPIDEMIOLOGY AND THERAPEUTIC POTENTIAL OF
PERSIAN SHALLOT (*Allium ascalonicum* L.) IN THE MANAGEMENT
OF METHICILLIN RESISTANT *Staphylococcus aureus* INFECTION**

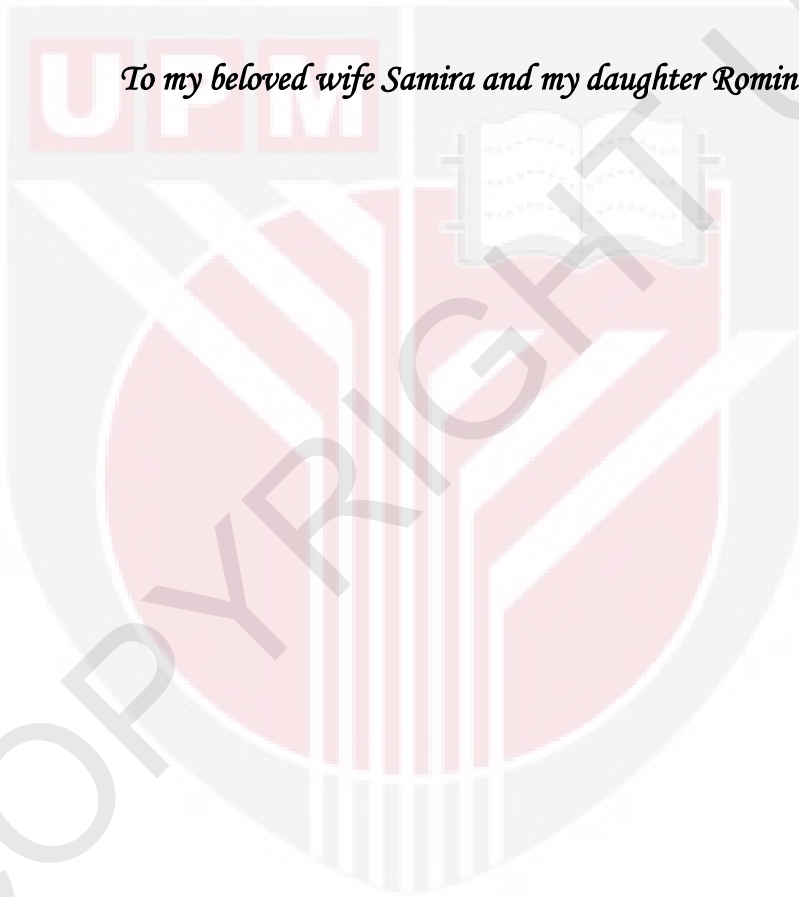


By

EHSANOLLAH GHAZNAVI RAD

**Thesis Submitted To School Of Graduate Studies, Universiti Putra Malaysia
In Fulfilment Of the Requirements for the Degree of Doctor of Philosophy**

October 2010



To my beloved wife Samira and my daughter Romina

© COPYRIGHT UPPM

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement of the degree of Doctor of Philosophy

MOLECULAR EPIDEMIOLOGY AND THERAPEUTIC POTENTIAL OF PERSIAN SHALLOT (*Allium ascalonicum* L.) IN THE MANAGEMENT OF METHICILLIN RESISTANT *Staphylococcus aureus* INFECTION

By

EHSANOLLAH GHAZNAVI RAD

October 2010

Chairman: Associate Professor Mariana Nor Shamsudin, PhD

Faculty: Medicine and Health Sciences

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an established human pathogen that causes both health care-associated (HA) and community-acquired (CA) infections. It has been shown that MRSA strains evolved from the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*) element carrying the *mecA* gene, which is responsible for methicillin resistance. Increased emergence of multidrug resistance among MRSA strains has become a major concern in the hospital environment, as it invokes a tremendous financial burden and enhanced morbidity and mortality due to hard-to-treat systemic infections.

MRSA was introduced into Malaysian hospital in the early 1970s; however the incidence rate has increased to more than 20% during the last few years. The efficient management of MRSA infection in any country relies on correct diagnosis,

understanding its antimicrobial resistance profile, epidemiology, transmission routes, appropriate therapeutics and the appropriate infection control measurements.

To achieve the goal of establishing baseline dataset for local clinical MRSA strains, Hospital Kuala Lumpur was focused in this study. A one year study from September 2007 to August 2008 was carried out on 389 isolates, a statistically calculated sample size. The prevalence of MRSA was found to be 44.1% and significantly higher in the patients of Indian ethnicity ($P < 0.001$).

Since the first step in any infection management is the correct diagnosis of etiological agents, in the current study a novel 9-valent multiplex PCR (MPCR) plus two primer pairs for *S. aureus* identification and detection of methicillin resistance was optimized with established primers by using reference strains. All 389 clinical MRSA isolates from Malaysia and 18 European isolates from the HARMONY collection harboring different SCCmec types were correctly characterized by the novel MPCR assay. Therefore the MPCR assay optimized here in could type any MRSA globally.

It is understood from the phenotypic antibiotic susceptibility testing that many of the local MRSA strains were multi drug resistant with different profiles indicating that more than one clone is circulating in the hospital. Hence additional epidemiological studies were clearly warranted in order to increase the insight into the dynamics of MRSA epidemiology in Malaysia. The molecular epidemiology of MRSA isolates were extensively investigated with multiple typing techniques.

In this study, molecular characterization of MRSA found that the majority (92.5%) of the isolates belonged to ST-239, *spa* type t037, and possessed the type III or IIIA *SCCmec*. Apart from this predominant clone, six (1.5%) isolates of ST-22, with two related *spa* types (t032 and t4184) and a singleton (t3213), carrying type IVh *SCCmec*, were detected for the first time in Asia. A limited number of CA-MRSA strains were also detected. These included ST-188/t189 (2.1%), ST-1/t127 (2.3%), and ST-7/t091 (1%). Current results revealed the predominance of ST-239-*SCCmec* III/IIIA and the penetration of ST-22 with different virulence gene profiles. The emergence in Malaysia of novel clones of known epidemic and pathogenic potential should be taken seriously.

In order to trace the source of nosocomial MRSA transmission in the hospital, the impact of HCWs and the hospital environment which could be potential MRSA transmission source were investigated. Of 460 HCWs participants, three (0.65%) were MRSA positive and among the 40 environmental samples four (10%) were found to be MRSA positive. From the current study it is understood that MRSA nasal carriage among HCWs is not the source of infection in the hospital, but hospital environment appears to pose a threat for nosocomial transmission, as all the strains isolated from the environment and clinical cases displayed a similar genetic background.

Probably the single most effective way of combating MRSA nosocomial infection is to improve hygiene in hospital environment and healthcare settings, in particular hand hygiene. When MRSA nosocomial infection is confirmed, measures to limit the spread of MRSA include following steps. First, it is necessary to put patients into isolation wards; in one part of a ward, with nursing by designated staff. Secondly, use of single-

bedded rooms is highly recommended. Finally, HCWs barrier precautions (gowns, gloves, masks, apron) as physical are barriers to transmission should be implemented.

An earlier study carried out in the laboratory has identified the DCM extract of *Allium ascalonicum* known as Persian shallot, to have surprisingly high antibacterial effect against *S. aureus* including MRSA with no cytotoxicity at MIC values of 2-4 mg/ml. In order to understand the actual mechanism of inhibition, transcriptome analysis using DNA-Microarray was performed. The investigation revealed that the extract specifically down-regulated the essential microbial fatty acid metabolizing FasII pathway. Because the human serum is full source of fatty acids, it was proven that the *A. ascolonicum* DCM extract was not suitable for systemic infection. However the result of topical application revealed that although the extract is not suitable for systemic use, it could be potent and safe for topical application.

These novel achievements made in this study will be of great value in the modern diagnostic, characterization and treatment. As the molecular system optimized could be readily applied in clinical diagnosis, source tracing assay would be effective in eradication and suppression of FASII through shallot application might be effective in local treatment of MRSA infection. The achievement of the current study is especially a significant contribution to the molecular epidemiology of MRSA in this central teaching hospital for determination of clonal relatedness and emergence of new clones. In addition the routine application of molecular system optimized in this study for the identification and typing of MRSA for epidemiological study definitely contribute toward early diagnosis of MRSA infection in clinical laboratories.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**EPIDIMIOLOGI MOLEKUL DAN POTENSI
RAWATAN BAWANG PARSI (*Allium ascalonicum* L.) DALAM
PENGURUSAN JANGKITAN *Staphylococcus aureus* RINTANG
METHICILLIN**

Oleh

EHSANOLLAH GHAZNAVI RAD

Oktober 2010

Pengerusi: Associate Professor Mariana Nor Shamsudin, PhD

Fakulti: Perubatan dan Sains Kesihatan

Staphylococcus aureus rintang terhadap methicillin (MRSA) merupakan patogen manusia yang mapan menyebabkan jangkitan berhubung kait dengan penjagaan kesihatan (HA) juga yang diperolehi daripada komuniti (CA). Telah ditunjukkan bahawa strem *S. aureus* yang pada asalnya rentan terhadap methicillin (MSSA) berubah menjadi strem MRSA melalui perolehan unsur kromosom kaset staphylococcal *mec* (SCC*mec*) yang membawa gen *mecA* dan bertanggungjawab bagi kerintangan terhadap methicillin. Kemunculan kerintangan-pelbagai ubat yang meningkat di kalangan strem MRSA telah menjadi suatu kebimbangan besar dalam persekitaran hospital kerana ianya mendatangkan beban kewangan yang amat tinggi serta kadar morbiditi dan kematian yang meningkat disebabkan jangkitan sistemik yang susah untuk dirawat.

MRSA diperkenalkan di Malaysia sejak awal 1970-an; namun kadar insiden telah meningkat lebih daripada 20% dalam beberapa tahun yang lepas. Pengurusan cekap

jangkitan MRSA di mana-mana negara bergantung kepada diagnosis tepat, pemahaman terhadap profil rintang antimikrob, epidimiologi, potensi membawa penyakit, cara penyebaran, dan rawatan yang bersesuaian.

Untuk mencapai matlamat bagi menetapkan maklumat garis-dasar untuk stren MRSA klinikal tempatan, kajian ini telah menjadikan Hospital Kuala Lumpur sebagai fokus utama. Kajian selama satu tahun bermula dari September 2007 ke Ogos 2008 telah dijalankan menggunakan 389 isolat, dengan saiz sampel yang ditentukan secara statistik. Didapati bahawa kadar kejadian MRSA adalah 44.1% dan lebih ketara dalam pesakit berbangsa India ($P < 0.001$).

Langkah pertama dalam mana-mana pengurusan jangkitan merupakan diagnosis tepat agen-agen penyebab dan dalam kajian ini PCR 'multiplex' (MPCR) 9-valens yang baru serta dua pasang primer untuk mengenalpasti *S. aureus* dan mengesan kerintangan terhadap methicillin telah dioptimumkan dengan menggunakan primer yang sudah mapan di dalam stren rujukan. Kesemua 389 isolat MRSA klinikal dari Malaysia dan 18 isolat Eropah dari koleksi HARMONY yang membawa jenis SCCmec yang berbeza telah dicirikan dengan tepat oleh esei MPCR baru ini. Kesemua isolat HARMONY juga telah dijeniskan dengan tepat. Ini menunjukkan esei MPCR yang dioptimumkan boleh menjeniskan setiap satu MRSA secara global.

Daripada ujian kerentanan antibiotik fenotip difahamkan bahawa banyak stren MRSA tempatan adalah rintang pelbagai ubat dengan profil yang berlainan menunjukkan terdapat lebih daripada satu klon yang sedang tersebar di dalam hospital. Maka, kajian

epidemiologi tambahan sememangnya diperlukan untuk meningkatkan pengetahuan bagi mendalami dinamik epidemiologi MRSA di Malaysia. Epidemiologi peringkat molekul isolat MRSA telah diselidik secara meluas dengan pelbagai teknik penjenisan.

Dalam kajian ini, pencirian molekul MRSA mendapati kebanyakan isolat (95.2%) adalah dari ST-239, *spa* jenis t037, dan mempunyai SCC*mec* jenis III atau IIIA. Selain daripada klon dominan ini, enam (1.5%) isolat ST-22, dengan dua jenis *spa* yang berhubungkait (t032 dan t4184) dan satu *spa* (t3213), yang membawa SCC*mec* jenis IVh, telah dikesan buat pertama kalinya di Asia. Sebilangan terhad strem komuniti (CA-MRSA) juga telah dikesan. Ini termasuk ST-188/t189 (2.1%), ST-1/t127 (2.3%), dan ST-7/t091 (1%). Keputusan terkini menunjukkan dominasi ST-239-SCC*mec* III/IIIA dan penembusan ST-22 dengan profil gen virulen yang berbeza. Kemunculan klon baru yang berpotensi secara epidemik dan patogenik di Malaysia harus diambil serius.

Dalam usaha menjejak sumber penularan MRSA di hospital, kesan yang dibawa pekerja kesihatan (HCW) dan persekitaran hospital yang berpotensi menjadi unsur penyebaran MRSA telah dikaji. Daripada 460 pekerja kesihatan yang turut serta, tiga (0.65%) adalah positif untuk MRSA, manakala dari kalangan 40 sampel persekitaran, empat (10%) didapati positif bagi MRSA. Dari kajian ini, difahamkan bahawa MRSA pembawaan hidung di kalangan pekerja kesihatan bukanlah sumber utama jangkitan di hospital yang dikaji, tetapi persekitaran hospital yang membawa risiko penyebaran hospital kerana kesemua strem yang diambil dari persekitaran dan dari kes-kes klinikal menunjukkan latarbelakang genetik yang serupa.

Sebuah kajian yang dijalankan sebelum ini di makmal telah mengenalpasti ekstrak DCM *Allium ascolanicum* yang disebut bawang Persia, mempunyai kesan antibakteria tinggi yang tidak dijangka terhadap *S. aureus* termasuklah MRSA tanpa menimbulkan kesan toksik kepada sel pada nilai MIC 2-4 mg/ml. Untuk memahami mekanisme sebenar rencatan, dalam kajian ini, analisis transkriptom menggunakan analisa 'DNA-Microarray' telah dijalankan. Penyelidikan ini menunjukkan bahawa ekstrak tersebut dengan khususnya menurun-laras laluan penting metabolisme asid lemak mikrob, FasII. Oleh kerana serum manusia kaya dengan asid-asid lemak, kajian ini telah membuktikan bahawa ekstrak DCM *A. ascolanicum* tidak sesuai untuk jangkitan sistemik; walau bagaimanapun penggunaannya untuk aplikasi luaran telah diterokai. Keputusan ujian kulit pula menunjukkan bahawa kepekatan asid lemak dalam kulit manusia adalah lebih rendah daripada dalam darah dan kepekatan rendah sebegini tidak dapat membekalkan keperluan asid lemak bakteria. Ditunjukkan juga bahawa walaupun ekstrak ini tidak sesuai untuk kegunaan sistemik, ianya berkesan dan selamat untuk aplikasi luaran.

Pencapaian baru yang diperoleh daripada kajian ini adalah amat bernilai dalam diagnosis, pencirian, dan rawatan moden. Oleh sebab sistem molekul yang dioptimumkan telah sedia untuk diaplikasi ke dalam diagnosis klinikal, kaedah jejak sumber akan berkesan dalam pembasmian dan penyekatan FASII melalui aplikasi shallot yang mungkin berkesan dalam rawatan tempatan bagi jangkitan MRSA.

Pencapaian daripada kajian ini merupakan sumbangan penting terutamanya kepada epidemiologi molekul MRSA dalam hospital pengajaran pusat ini untuk mengenalpasti hubungkait klonal dan kemunculan klon baru. Tambahan pula, aplikasi rutin sistem molekul yang dioptimumkan dalam kajian ini untuk pengenalpastian dan penjenisan

MRSA bagi kajian epidemiologi semestinya dapat menyumbang kepada pengenalpastian awal jangkitan MRSA di dalam makmal klinikal.



ACKNOWLEDGEMENTS

Many thanks to God, I have completed writing this thesis but of course with the help and support from fantastic peoples around me. First and foremost, I wish to express my heartfelt gratitude to my advisor, Associate Professor Dr. Mariana Nor Shamsudin for her professional guidance and support in academic and in real life. I am very indebted to her patience and invaluable advices that inspired me to see things positively and felt honored with her confidence and trust on my ability.

It has been an honor and pleasure to have Dr Vasanthakumari Neela as a member of supervisory committee.

I would like to express my deepest thanks and admiration to Associate Professor Dr Chong Pei Pei and Associate Professor Dr. Zamberi Sekawi for serving in my graduate committee.

My sincere thank to group of eminent scientists: Professor Alex van Belkum, Mrs Mehri Tavakol, Dr Willem van Wamel, Dr Nicole Lemmens den Toom in Erasmus Medical for their kindness and support for the part of the work was done in their lab.

Last but not the least, I owe my loving thanks to my wife Samira Aminzadeh, my daughter Romina, my parents, Father and Mother in law , my dear brothers Dr Ali and Dr Peyman Ghaznavi Rad for their support, understanding and encouragement.

I certify that an Examination Committee met on date of viva voce to conduct the final examination of Ehsanollah Ghaznavi Rad on his Doctor of Philosophy thesis entitled Management of Methicillin Resistant *Staphylococcus aureus* Infection Through Molecular Epidemiology and Potential Therapeutics Determination in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are follows:

Hejar Abdul Rahman

Assiat Professor
Department of Community Health
Faculty of Medicine and Health Sciences
University Putra Malaysia
(Chairman)

Raha Abdul Rahim

Professor
Department of Cell & Molecular Biology (Head)
Faculty of Biotechnology and Biomolecular Sciences
University Putra Malaysia
(Member)

Saleha Abdul Aziz, PhD

Professor
Department of Veterinary Pathology & Microbiology
Faculty of Veterinary Medicine
University Putra Malaysia
(Member)

Willem van Leeuwen

Proffesor
University of Applied Sciences
Zernikedreef 11, 2333 CK Leiden
The Netherlands
(External Examiner)

SHAMSUDDIN SULAIMAN, PhD

Professor/ Deputy Dean
School of Graduate Studies
niversity Putra Malaysia

Date:

This thesis was submitted to senate of Universiti Putra Malaysia and has been accepted as fulfilment of requirement for degree of Doctor of Philosophy. Members of the Supervisory Committee were follows:

Mariana Nor Shamsudin, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

VasanthaKumari Neela, PhD

Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Chong Pei Pei, PhD

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

Zamberi Bin Sekawi, MD, MPath

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

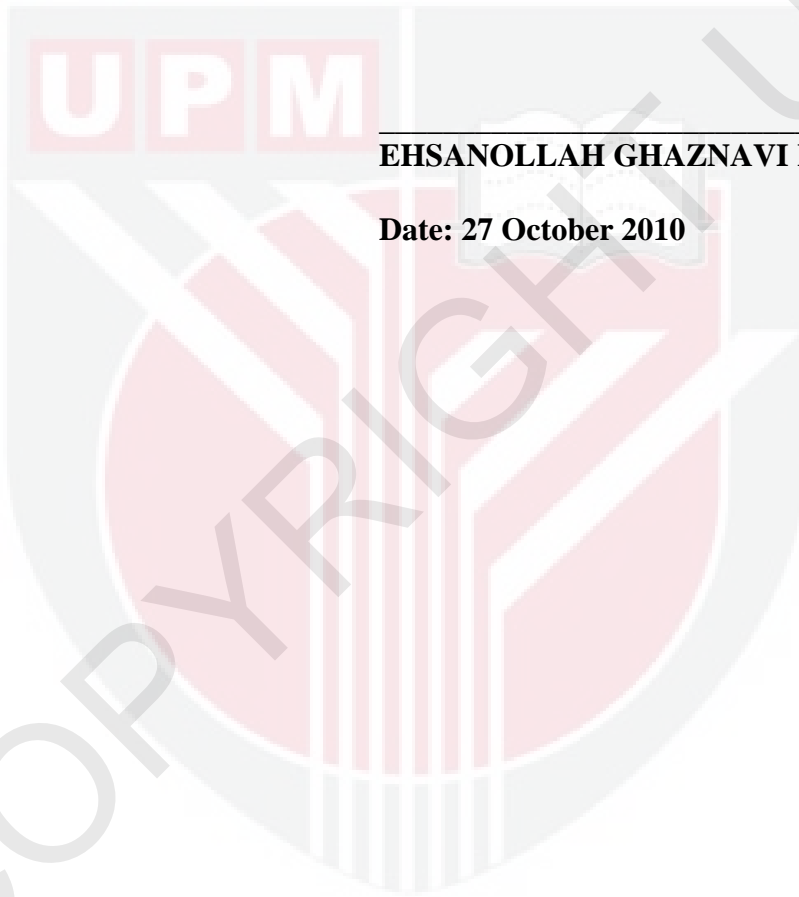
HASANAH MOHD. GHAZALI, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for other degree at Universiti Putra Malaysia or other institutions.



EHSANOLLAH GHAZNAVI RAD

Date: 27 October 2010



TABLE OF CONTENTS

ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGMENTS	xii
APPROVAL	xiii
DECLARATION	xv
LIST OF TABLES	xx
LIST OF FIGURES	xxi
ABBREVIATIONS	xxiv

CHAPTER

1	GENERAL INTRODUCTION	1
	1.1 Introduction	1
	1.2 Thesis Organisation	7
2	LITERATURE REVIEW	8
	2.1 Introduction	8
	2.2 Infectious Diseases	8
	2.3 Family of Staphylococcaceae	9
	2.3.1 Genus <i>Staphylococcus</i>	9
	2.3.2 Species <i>Staphylococcus</i>	10
	2.3.3 Subspecies <i>Staphylococcus</i>	10
	2.4 <i>Staphylococcus aureus</i>	11
	2.5 Emergence of MRSA	12
	2.6 Origin of SCCmec gene	13
	2.7 Molecular structure of SCCmec	15
	2.8 SCCmec typing	18
	2.9 Evolution of HA-MRSA	19
	2.10 Evolution of CA-MRSA	21
	2.11 Nasal carriage of MRSA	23
	2.12 Methicillin resistant <i>Staphylococcus aureus</i> pathogenicity	23
	2.13 Antibiotic Resistance in MRSA	26
	2.14 Molecular epidemiology of MRSA	26
	2.14.1 Multilocus Sequence Typing (MLST)	30
	2.14.2 <i>Staphylococcus aureus</i> Protein A typing (<i>Spa</i> typing)	32
	2.14.3 Pulse Field Gel Electrophoresis	34
	2.14.4 Direct repeat typing (<i>dru</i>)	35
	2.15 Comparison and application of different typing method	35

2.16	Epidemiology of MRSA	36
2.16.1	Staphylococcal source and transmission cycle	36
2.16.2	Contamination in hospitals	38
2.16.3	Survival of staphylococci	39
2.17	Methicillin-resistant <i>Staphylococcus aureus</i> as a new zoonotic agent	39
2.18	Worldwide distribution of MRSA clones and status in Malaysia	41
2.19	Virulence Factor	43
2.19.1	Cytotoxins and protein enzymes	45
2.19.2	Superantigenic toxin	46
2.19.3	Arginine catabolic mobile elements (ACME)	49
2.19.4	Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)	50
2.19.5	Biofilm associated proteins	50
2.19.6	Accessory gene regulator system (<i>agr</i>)	51
2.20	Alternative Treatment for MRSA	52
2.20.1	<i>Allium ascallenicum</i> as Anti MRSA Herb	53
2.20.2	Role of DNA Microarray in antibacterial mechanism determination	58
2.20.3	Application of Microarray	59
3	A SIMPLIFIED MULTIPLEX PCR ASSAY FOR FAST AND EASY DISCRIMINATION OF GLOBALLY DISTRIBUTED SCCMEC TYPES IN METHICILLIN RESISTANT <i>STAPHYLOCOCCUS AUREUS</i>	63
3.1	Introduction	63
3.2	Materials and Methods	65
3.2.1	Bacterial isolates:	65
3.2.2	Multiplex PCR:	70
3.2.3	Validation:	71
3.3	Results	71
3.4	Discussion	75
4	PREDOMINANCE AND EMERGENCE OF CLONES OF HOSPITAL ACQUIRED METHICILLIN RESISTANT <i>STAPHYLOCOCCUS AUREUS</i> IN MALAYSIA	78
4.1	Introduction	78
4.2	Materials and Methods	81
4.2.1	Clinical setting and bacterial strains	81
4.2.2	Confirmation of isolates by conventional biochemical tests	82
4.2.3	Molecular characterization of methicillin resistant <i>S. aureus</i> isolates	85
4.2.4	To establish the virulent gene profiles of MRSA isolates	91
4.2.5	Statistical analysis	92
4.3	Results	93
4.3.1	Demographical characterization of MRSA strains	93
4.3.2	SCC <i>mec</i> typing	95

4.3.3	Detection of <i>spa</i> gene in MRSA isolates	96
4.3.4	Multi-locus sequence typing (MLST) of MRSA	99
4.3.5	<i>agr</i> typing	102
4.3.6	Toxin genes profiles	103
4.3.7	Detection and prevalence of Enzymes and Cytotoxin	113
4.3.8	Detection of enhanced growth and development factor	114
4.3.9	Detection and prevalence of MSCRAMMs	115
4.3.10	Detection of biofilm associated proteins (BAP)	117
4.3.11	Toxin genes profiles with association to virulence genes and clones	119
4.4	Discussion	121
5	ANTIBIOTIC RESISTANCE PROFILE, DETERMINATION OF GENETIC DIVERSITY BY PFGE METHOD AND DRU TYPING OF MRSA ISOLATES FROM CLINICAL SAMPLES IN TERTIARY HOSPITAL	127
5.1	Introduction	127
5.2	Materials and Methods	131
5.2.1	Antimicrobial susceptibility	131
5.2.2	MLS _B resistant gene investigation	133
5.2.3	PFGE	134
5.2.4	Direct Repeat Unit (<i>dru</i>) typing	135
5.3	Result	137
5.3.1	Antimicrobial susceptibility results	137
5.3.2	Determination of MLS _B resistant gene	140
5.3.3	Molecular typing	143
5.3.4	Comparison of typing method	147
5.4	Discussion	148
6	HOSPITAL ENVIRONMENT CONTAMINATION AS A SOURCE OF NOSOCOMIAL INFECTION WITH METHICILLIN-RESISTANT <i>STAPHYLOCOCCUS AUREUS</i>	158
6.1	Introduction	158
6.2	Materials and Methods	161
6.2.1	<i>spa</i> typing	162
6.2.2	MLST typing	162
6.2.3	SCC <i>mec</i> typing	162
6.2.4	PFGE	162
6.2.5	Virulence gene determination	163
6.3	Results	163
6.4	Discussion	169
7	A MIXED- COMPOUND DICHLOROMETHANE EXTRACT OF PERSIAN SHALLOT (<i>ALLIUM ASCALONICUM</i>) EXERTS IN	

	VITRO AND TOPICAL ANTI STAPHYLOCOCCAL ACTIVITY VIA FSII INTERFERENCE	174
7.1	Introduction	174
7.2	Materials and methods	176
	7.2.1 Plant collection	176
	7.2.2 Extraction	177
	7.2.3 Processing of extracts	178
	7.2.4 Bacterial strains	178
	7.2.5 Disk diffusion tests	178
	7.2.6 Minimum inhibitory concentration (MIC) assay	179
	7.2.7 Time killing study	179
	7.2.8 Cytotoxicity assay on Vero cell lines	180
	7.2.9 Transcriptome analysis using DNA arrays	181
7.3	Results	185
	7.3.1 Anti-bacterial activity	185
	7.3.2 Minimum Inhibitory Concentrations of the extract	186
	7.3.3 Time-kill study	187
	7.3.4 Cytotoxicity assay on Vero cell lines	188
	7.3.5 Transcriptomic analyses using DNA arrays	188
	7.3.6 Topical application of the extract	191
7.4	Discussion	193
8	SUMMARY, GENERAL CONCLUSION AND RECOMMENDATIONS	197
	8.1 Summary and General Conclusion	197
	8.2 Recommendations	200
	REFERENCSS	204
	APPENDICES	238
	BIO DATA OF STUDENT	258
	LIST OF PUBLICATIONS	259