UNIVERSITI PUTRA MALAYSIA

UTILIZATION OF MOLECULAR SYSTEMS FOR THE IDENTIFICATION AND TYPING OF CLINICALLY RELEVANT MULTIPLE DRUG RESISTANT *STAPHYLOCOCCUS AUREUS*

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By

VASANTHAKUMARI NEELA

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

January 2005

Dedicated to my husband, daughter, son and my parents
For their strength and courage.

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

UTILIZATION OF MOLECULAR SYSTEMS FOR THE IDENTIFICATION AND TYPING OF CLINICALLY RELEVANT MULTIPLE DRUG RESISTANT *STAPHYLOCOCCUS AUREUS*

By

VASANTHA KUMARI NEELA

January 2005

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Staphylococcus aureus is an important human nosocomial pathogen that can cause a variety of skin infections and toxin-mediated diseases including gastroenteritis, staphylococcal scalded-skin syndrome and toxic shock syndrome. Whilst the use of antibiotics can kill most strains of pathogenic microbes, the increase of multiple drug resistant *S. aureus*, especially among hospital patients have been a worrying trend. In order to treat patients infected with this organism at the earliest time possible and to prevent further nosocomial outbreaks, it is necessary to rapidly identify the multiple drug resistant *S. aureus*, so that the patient can be treated with the correct antibiotic on time and thus prevent further complications.

S. aureus besides being a nosocomial pathogen causing frequent nosocomial infections also causes community acquired outbreaks. The increased prevalence of *S. aureus* infections may be prevented if the epidemiology of *S. aureus* is studied, whereby the

spread of outbreak clones can be identified and treated with the appropriate drugs. Therefore, the aim of this study was to investigate suitable molecular systems for use in the rapid identification of *S. aureus* and detection of multiple drug resistant *S. aureus* and for typing of strain variations for epidemiological understanding of *S. aureus* in Malaysia.

In this study, a total of eighty-nine clinical *S. aureus* isolates obtained from five different hospitals in Malaysia and from one pathology laboratory were studied. All the isolates were confirmed for *S. aureus* by the presence of species-specific Sa442 fragment (*S. aureus* specific fragment). Sequencing of the Sa442 fragment from isolates obtained from the different geographical locations identified this fragment as an epidemiological marker as it showed a wide variation of 1-10 % in the nucleotide sequences among the *S. aureus* isolates studied.

For rapid identification of *S. aureus* and detection of multiple drug resistant strains, two molecular assays were utilized. In the first assay, a multiplex PCR based strategy was used, whereby the genes responsible for methicillin (*mecA*), mupirocin (*iles2*), gentamycin (*aac(6')-aph(2")*), erythromycin(*ermA*) resistance and species specific Sa442 fragment were amplified. Results of the assay indicated the amplification of antibiotic resistant genes and Sa442 fragment at the expected sizes of 533, 456, 174, 139 and 108bp respectively. All the amplified products were further confirmed by sequencing. The second assay was a membrane assay based on an optimized dot blot hybridization technique. In the dot blot assay, a set of oligonuclotide probes designed from the antibiotic resistance genes and the Sa442 fragment sequences were highly sensitive and

specific for the respective bacterial target genes. The membrane assay developed was able to detect multiple drug resistant *S. aureus* isolates in less than two hours after obtaining pure culture isolates. Detection is also possible in less than two hours from spiked urine and blood samples, as well as for direct nasal samples. The results obtained with both polymerase chain reaction (PCR) and membrane assay were found to be similar whereby, 58.8, 67.7, 97.7 and 1.1% of the isolates carried *mecA, aac(6')-aph(2")*, *ermA* and *iles-2* genes respectively. The overall correlation between the antibiotic resistance (disc diffusion test) and presence of antibiotic resistant genes (PCR and membrane assay) were found to be 77.3% for methicillin, 73.7 % for gentamycin, 95.4% for erythromycin and 100% mupirocin.

The molecular epidemiology of local *S. aureus* was studied using randomly amplified polymerase chain reaction (RAPD) and repetitive element sequence based PCR (rep-PCR). Four out of the 20 arbitrary primers screened were highly efficient for use in molecular typing of *S. aureus* isolates. The rep-PCR typing primers designed from the staphylococcal repetitive sequences (STAR) were also markedly feasible for typing of *S. aureus* isolates. The RAPD study for molecular epidemiology showed wide variation in *S. aureus* isolates, as seen by genetic distance value based on Jaccard's index ranged from 0.037 to 0.954545. Similarly, in rep-PCR study, a wide variation in genetic distance value based on Jaccard's index ranged from 0.037037 to 0.894737. A wide variation in genetic distance was seen in the clonal diversity of local *S. aureus* isolates, where by, isolates were divided into four (4) clones namely Miri, Kuantan, Kota Bharu and Seremban, with Miri as the most predominant clone. In addition, RAPD was able to

distinguish between methicillin resistant *Staphylococcus aureus* (MRSA) and non-MRSA isolates, showing the spread of two MRSA clones in Malaysia. RAPD analysis produced two (2) molecular markers, at positions 500 bp with primer OPAE 14 and 750 bp with OPAE 15, whereas, rep-PCR produced three (3) molecular markers, positions 500 with rep primer1, between 1500 and 2000 bp with rep 2, and slightly above 750 bp with rep primer 3, in most of the *S. aureus* isolates studied. From the five (5) markers obtained with RAPD and rep-PCR, the putative 500 bp rep marker was cloned in PCR 2.1 Topo vector and sequenced. The 500 bp rep marker was selected, as this marker was obtained through the amplification of *S. aureus* isolates with the primer designed from *S. aureus* genome and also because of the small size. The sequence obtained identified the rep-marker as a 489 bp fragment, showing 95% homology to a region in glyceraldehydes –3-phosphate dehydrogenase (GAP) operon in *S. aureus* genome, whose coding potential is unknown. The higher percentage (95%) similarity of the rep marker to *S. aureus* genome emphasized the importance of the rep marker in species-specific identification.

To investigate the potentiality of the rep marker in species-specific identification of *S. aureus* isolates, a PCR and a membrane assay were developed with primers and probe designed from the rep sequence. The PCR and membrane assay showed positive signal for all eighty-nine *S. aureus* isolates tested and no signal was seen for other Gram-positive and Gram-negative species tested, appreciating the specificity and the sensitivity of rep primers and probes in species-specific detection of *S. aureus* isolates. Sequencing of the rep marker from isolates obtained from different geographical locations, identified this marker (rep) as a potential diagnostic marker as it is highly conserved in *S. aureus*

genome showing 98-99% sequence similarity among the isolates. Besides for diagnosis, using two typing procedures (RAPD and rep-PCR) to study the clonal relatedness among the local *S. aureus* isolates which were developed with suitable RAPD and rep primers, they were able to correctly type *S. aureus* isolates according to the geographical location and also to differentiate between MRSA and non-MRSA. Although the RAPD primers are commercially available, the rep primers identified are still not published for use in typing *S. aureus*. The patterning of the novel rep primers and probe for typing and diagnosis of *S. aureus* will be extremely useful in the clinical diagnosis as the primers and probes innovated are highly specific and ubiquitous in all *S. aureus* isolates.

These novel achievements made in the study will be of great value in the modern diagnostic era as the molecular systems optimized could be readily applied in the clinical diagnosis due to the specificity and rapidity in the detection of multiple drug resistant *S. aureus*. The achievements of the current study is especially a significant contribution to the clinical diagnostics and infectious disease research because the utilization of the optimized system incorporated as diagnostic kit will enhance the sensitivity and rapidity of molecular based detection in combination with sub-typing ability. Therefore, the routine application of the molecular systems optimized and the primers and probes developed in this study for the rapid identification of multiple drug resistant *S. aureus* and to study the epidemiology of *S. aureus* will definitely contribute towards early diagnosis of *S. aureus* infection in clinical laboratories. The epidemiological investigation will aid in developing more effective strategies in preventing and controlling the further spread of multiple drug resistant *S. aureus* clones in Malaysia.

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

PENGGUNAAN SISTEM MOLEKUL BAGI MENGENALPASTI DAN MENGKELASKAN *STAPHYLOCOCCUS AUREUS* KLINIKAL YANG RINTANG PELBAGAI UBAT

Oleh

Vasantha Kumari Neela

Januari 2005

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Staphylococcus aureus merupakan salah satu patogen yang kerap menyebabkan infeksi kulit dan penyakit lain termasuk gastroenteritis, sindrom kulit terbakar, dan sindrom kejutan toksik. Walaupun penggunaan antibiotik boleh membunuh kebanyakkan bakteria patogenik, namun peningkatan rintangan terhadap *S. aureus* tetap berlaku terutama di kalangan pesakit hospital. Bagi merawat pesakit yang dijangkiti dengan organisma ini pada peringkat awal, amatlah penting bagi mengenalpasti kepelbagaian ubat yang rintang terhadap *S .aureus*, agar pesakit boleh dirawat dengan antibiotik yang betul, tepat pada masanya dan bagi menghalang komplikasi seterusnya. *S. aureus* juga adalah patogen nosocomial yang menyebabkan wabak nosocomial yang kerap merebak kepada komuniti. Perkara ini boleh dicegah jika epidemiologi *S. aureus* dikaji, dari segi mengenalpasti perebakan klon dan dengan ini ubat yang sesuai boleh digunakan untuk rawatan. Oleh itu, tujuan kajian ini adalah untuk membentuk satu sistem molekul yang pantas bagi

mengenalpasti kerintangan kepelbagaian antibiotik terhadap *S. aureus* serta mengkaji epidemiologi *S. aureus* di Malaysia.

Di dalam kajian ini, sejumlah lapan puluh sembilan isolat klinikal *S.aureus* daripada lima hospital yang berbeza di Malaysia dan daripada satu makmal patologi digunakan. Semua isolat disaring bagi kehadiran spesis spesifik Sa442 yang mengesahkan ia adalah isolate *S. aureus.*

Bagi pengenalpastian kerintangan kepelbagaian antibiotik terhadap *S. aureus*, dua sistem molekul telah digunakan iaitu 'multiplek PCR', di mana gen yang bertanggungjawab untuk rintangan terhadap antibiotik *methicillin, gentamycin, mupirocin* dan *erythromycin* diamplifikasikan. Keputusan menunjukkan amplifikasi gen yang rintang terhadap antibiotik adalah seperti yang dijangkakan iaitu pada 533, 456, 174, 139 dan 108 bp. Semua produk gen yang diamplifikasikan disahkan dengan penentuan jujukan gen. Kemudian assai membran yang berdasarkan teknik hibrid "dot blot" digunakan untuk mengenalpasti kerintangan kepelbagaian antibiotik *S. aureus*. Set prob oligonukleotida dibentuk daripada antibiotik yang rintang dan jujukan fragmen Sa442 diuji di dalam assai membran. Sensitiviti dan kespesifikan prob yang dikaji menunjukkan prob yang dibentuk adalah sensitif dan spesifik kepada sasaran tertentu. Assai membran yang dibentuk didapati boleh mengesan kepelbagaian antibiotik rintang dalam masa kurang daripada dua jam tanpa memerlukan instrumen yang mahal. Hasil yang didapati dengan PCR dan assai membran didapati sama, di mana 58.8% daripada isolatnya mempunyai ketahanan terhadap methicillin, 67.7% terhadap gentamycin, 97.7% terhadap erythromycin dan

1.1% terhadap mupirocin. Korelasi keseluruhan di antara kerintangan antibiotik dan kehadiran gen-gen rintang antibiotik adalah 77.3% untuk methicillin, 73.7% untuk gentamycin, 95.4% untuk erythromycin, dan 100% untuk mupirocin.

Epidemiologi molekul *S.aureus* tempatan dikaji dengan menggunakan dua kaedah rawak iaitu teknik amplifikasi rantai reaksi polimerase (RAPD) dengan empat primer OPAE 06, 10, 14 dan 15 dan teknik elemen jujukan berulang yang berasaskan teknik PCR (rep-PCR) dengan primer yang didapati daripada jujukan berulang staphylococcal (STAR). Kajian RAPD bagi epidemiologi molekul menunjukkan variasi yang meluas bagi isolate *Staphylococcus aureus,* seperti yang dilihat dari nilai jarak genetik berdasarkan Indeks Jaccard dalam kadar di antara 0.037 ke 0.954545. Seperti juga dalam kajian rep-PCR, variasi meluas dalam nilai jarak genetik berdasarkan Indeks Jaccard dengan kadar dari 0.037037 ke 0.894737. Variasi meluas dalam jarak genetik dilihat dalam kepelbagaian klonal bagi isolate *S.aureus* tempatan di mana isolate dibahagikan kepada empat (4) klon iaitu Miri, Kuantan, Kota Bharu dan Seremban, dengan Miri sebagai klon yang paling dominan. Tambahan pula, RAPD dapat membezakan antara isolat *S.aureus* yang rintang terhadap methicillin (MRSA) dan isolat bukan MRSA, menunjukkan penyebaran dua klon MRSA di Malaysia. RAPD menghasilkan dua penanda molekul pada posisi 500 dengan primer OPAE 14 dan 750 bp dengan primer OPAE 15, melalui teknik rep-PCR pula tiga penanda molekul dihasilkan pada posisi 500 dengan primer rep 1, antara 1500 dan 2000 bp dengan primer rep 2, kurang sedikit daripada 750 bp dengan primer rep 3, pada kebanyakkan isolates *S. aureus* yang dikaji. Daripada lima penanda molekul yang digunakan penanda 500 bp rep di klonkan ke dalam Topo 2.1 vektor dan di jujukan.

Jujukan penanda 500bp menunjukkan 95% homologi pada bahagian operon glyseraldehid-3-fosfat dihidrogenase (GAP) dalam *S. aureus* genome. Peratusan yang tinggi (95%) yang mana sama dengan penunjuk rep pada genome *S. aureus* menunjukkan kepentingan penunjuk rep untuk digunakan sebagai pengenalpastian spesifik spesis.

Bagi membuktikan penunjuk rep boleh digunakan dalam pengenalpastian spesifik spesis isolat *S. aureus,* PCR dan assai membrane dicipta dengan penggunaan primer dan prob yang dibina daripada jujukan rep. Hasilnya ,kedua-dua teknik menunjukkan signal yang positif kepada semua lapan puluh sembilan isolate *S. aureus* yang digunakan dan tidak pada spesis gram positif dan gram negatif yang lain. Jujukan penunjuk rep daripada isolate yang diperolehi daripada lokasi georafi yang berbeza, menunjukkan penunjuk ini (rep) berpotensi sebagai penunjuk diagnostik disebabkan ia didapati sama dikalangan isolat *S. aureus* (98%-99%). Selain daripada diagnostik, dua kaedah pengkelasan (RAPD and rep-PCR) bagi mengkaji perkaitan antara klon-klon diantara isolate *S. aureus* dikenalpasti, yang mana pengkelasan isolat *S. aureus* berdasarkan lokasi geografi dan perbezaan antara MRSA dan bukan MRSA dapat dilakukan dengan tepat. Walaupun primer RAPD boleh didapati secara komersil, primer rep yang dikenalpasti masih belum diterbitkan bagi pengkelasan *S.aureus.* Pencorakan bagi primer rep dan probe baru untuk pengelasan dan diagnosis *S.aureus* akan menjadi sangat berguna dalam diagnosis klinikal kerana primer dan probe yang direka adalah sangat spesifik dan didapati dalam semua isolate *S.aureus*.

Kajian ini membolehkan diagnostik baru iaitu dengan menggunakan kaedah molekular diaplikasikan dalam diagnosis klinikal disebabkan kespesifikan dan kepantasan dalam mengesan kerintangan kepelbagaian antibiotik *S. aureus*. Pencapaian kajian ini boleh menyumbang dalam diagnostik klinikal dan kajian penyakit kerana penggunaan sistem ini dalam bentuk 'kit' diagnostik akan meningkatkan sensitiviti dan kepantasan pengesanan molekular yang bergabung dengan kemampuan pengkelasannya. Oleh itu, penggunaan kerap sistem molekul ini dalam mempercepatkan pengenalpastian kerintangan kepelbagaian antibiotik *S. aureus* dan bagi mengkaji epidemiologi bakteria dapat menyumbang kepada diagnostik awal jangkitan *S. aureus* dalam makmal klinikal. Siasatan epidemiologi boleh menyumbang dalam penghasilan strategi yang lebih efektif dalam pencegahan dan pengawalan perebakan klon *S. aureus* rintang kepelbagaian antibiotik di Malaysia.

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I certify that an Examination Committee has met on 17th January 2005 to conduct the final examination of Vasantha Kumari Neela on her Doctor of Philosophy thesis entitled "Utilization of Molecular Systems for the Identification and Typing of Clinically Relevant Multiple Drug Resistant *Staphylococcus aureus*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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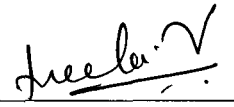
DECLARATION

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 Universiti Putra Malaysia or other institutions.



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