UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND CHARACTERIZATION OF BIOFILM-PRODUCING CLINICAL ISOLATES OF Staphylococcus aureus

SALMAN SAHAB ATSHAN

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DOCTOR OF PHILOSOPHY
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IDENTIFICATION AND CHARACTERIZATION OF BIOFILM-PRODUCING CLINICAL ISOLATES OF *Staphylococcus aureus*

By

SALMAN SAHAB ATSHAN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the requirements for the degree of Doctor of Philosophy

June 2013

DEDICATION

To my parents, daughter, son and my wife for invaluable support and extraordinary courage

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

IDENTIFICATION AND CHARACTERIZATION OF BIOFILM-PRODUCING CLINICAL ISOLATES OF *Staphylococcus aureus*

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SALMAN SAHAB ATSHAN

June 2013

Chairman: Prof. Mariana Nor Shamsudin, PhD

Faculty: Medicine and Health Sciences

Staphylococcus aureus is considered the major etiological agent of human infections. It is a biofilm-forming bacterium, which embedded itself in a matrix of extracellular polysaccharide (slime), and facilitates the adherence of these microorganisms to biomedical surfaces causing many persistent infections. The main issue with biofilm has become a global public health problem that is impacted by the insufficient management of patients infected with biofilm growth as extremely adaptable to antibiotic pressure. The ability of *S. aureus* to form biofilm is a long-known fact but the problem involving the issue of biofilm identification has remained since the availability of the phenotypic approach of growth on highly selective or differential media can provide identification of biofilm formation but with a high margin of error through many false negative outcomes. In line with these shortcomings, the present study embarked on

several strategies to overcome the issue of inaccurate biofilm identification through the development of an improved method that can provide positive identification. In this study, it was found that our modified-Congo red agar was significantly different from published-CRA ($P < 0.05$). The modified agar constituents provided not only stable 100% formation of black, also showed very high correlation with standard methods and with the presence of *ica*ADBC biofilm genes. In the second part of the work, the ability to adhere and produce biofilms of genotypically different clones of *S. aureus* was characterised. The study found the isolates that belonging to similar *spa*, SCCmec and ST types have similar abilities to produce biofilms. Moreover, isolates that have different *spa* types showed high variation in their ability to produce biofilms.The results indicate that differences in biofilm production capacities are caused by the differences in surface protein A (*spa*) type and are not due to differences in MLST and SCCmec types. In the third part of the work, the prevalence and distribution of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and biofilms genes in different clones of *S. aureus* were determined. the study found *ica*ADBC, *fnb*A, *eno, ebp*s, *clf*A and *clf*B genes to be present with a high prevalence and were equally distributed between the various clone types of 60 MSSA and MRSA clinical isolates, while the prevalence of other MSCRAMM genes were found to be variable. In the fourth part of the work, the transcriptional profiles of specific staphylococcal genes encoding MSCRAMMS and *ica*ADBC were determined during gradual changes in complexity of the biofilm production under different growth phases. The results indicate that the relative expression of MSCRAMMS and *ica*ADBC genes in comparison with the phenotypic biofilm morphology can be utilized as a model to study the up- and down- regulation of such genes. Delayed expression of certain genes during stationary phase biofilms grown at significantly higher

levels are considered important for biofilm development and for the survival of composing cells in a nutrient-scarce niche. In the fifth part of this work, the extracellular 2DE protein profiles among genotypically different clone types and under different time-points of biofilm developed growth of *S. aureus* were characterized. The main results of 2DE studies showed a high degree of extracellular protein heterogeneity among the various clone types and under different time-points growth, indicating that different regulation modes of growth processes are operating under different clone types and under altered time conditions. In the sixth part of this work, the antimicrobial susceptibility patterns (glycopeptide, β-lactam, lipopeptide, oxazolidinones and glycylcycline) of different *S. aureus* clone types were determined. The results revealed that MICs and the bactericidal activities of these agents' classes within the different *spa* types were largely different. However, the MIC and MBC among clones within the same *spa* and MLST type were slightly different. Moreover, the minimum biofilm reduction concentrations (MBRCs) of these agents in the prevention of biofilm formation *in vitro* were overall greater than the CLSI-defined planktonic MIC breakpoint for resistance and quite variable among different clone types. The diversity in the antibiotic susceptibilities of isolates within the various clone types emphasises the need for continuous monitoring for the clones and clinicians should consider a correct antibiotic rather than empirical treatment. In the last part of this study, the effect of sub-inhibitory concentrations of vancomycin and tigecycline on the steady-state mRNA transcription levels of MSCRAMM and the *ica*ADBC target gene, as well as on secretion of exoproteins of different clone types of *S. aureus* isolates were studied. The results indicate that the effects of these antibiotics generally affecting all virulence factors of selected target genes and the secretion of exoproteins. Thus might enhance

the virulence of this bacterium, therefore using these antibiotics to treat *S. aureus* infections may contribute to unpredictable results.

Conclusion: We conclude that a considerable difference exists among similar and various clone types of *S. aureus.* This variation could have contributed to the degree of virulence even within the same clone and enhanced heterogeneity in the infection potential. Thus, new genetic diversity suggests that the development of a rapid and precise identification profile for each clone type in human infections is very important to prescribe appropriate antibiotics and reduce the empirical treatment.

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

PENGENALPASTIAN DAN PENCIRIAN *Stapylococcus aureus* PENGHASIL BIOFILEM DARI ISOLAT KLINIKAL

Oleh

SALMAN SAHAB ATSHAN

Jun 2013

Pengerusi: Prof. Mariana Nor Shamsudin, PhD

Fakulti: Perubatan dan Sains Kesihatan

Staphylococcus aureus dianggap ejen etiological utama jangkitan manusia.Ia adalah bakteria biofilm-membentuk, yang tertanam sendiri dalam matriks polisakarida extracellular (lendir), dan memudahkan pematuhan ini mikroorganisma pada permukaan bioperubatan menyebabkan banyak jangkitan berterusan. Isu utama dengan lapisan yang telah menjadi satu masalah kesihatan awam global yang dipengaruhi oleh pengurusan yang tidak mencukupi pesakit yang dijangkiti dengan pertumbuhan biofilm sebagai sangat cepat menyesuaikan diri kepada tekanan antibiotik.Kebolehan *S. aureus* untuk menghasilkan biofilem telah lama diketahui namun masalah yang melibatkan penentuan biofilem masih wujud disebabkan kaedah penentuan berdasarkan fenotipik memerlukan medium yang sangat spesifik dan berbeza. Kaedah ini berupaya menentukan biofilem namun kadar kesalahan adalah tinggi disebabkan keputusan

yang salah-benar. Justeru, sejajar dengan keperluan yang sangat tinggi, kajian ini mengemukakan beberapa strategibagi mengatasi isu pengenalan biofilm tepat melalui pembangunan kaedah yang lebih baik yang boleh memberikan pengenalan positif.Keputusan yang didapati daripada CRA yang telah diubahsuai dalam kajian ini adalah berbeza secara signifikan daripada CRA yang telah diterbitkan ($P<0.05$). Kandungan agar yang diubahsuai bukan sekadar menghasilkan 100% pigmen hitam yang stabil malah menunjukkan hubungkait yang sangat tinggi dengan kaedah piawai dan kehadiran gen biofilem *ica*ADBC. Bahagian kedua kajian**,** ini melibatkan pencirian kebolehan pelbagai klon *S. aureus* yang berbeza secara genotipik untuk melekat dan menghasilkan biofilem. Kajian ini telah menunjukkan bahawa pencilan yang mempunyai jenis spa, SCCmec dan MLST yang sama mempunyai kebolehan yang sama untuk menghasilkan biofilem. Tambahan pula pencilan yang mempunyai jenis spa yang berbeza menunjukkan variasi yang tinggi dalam kebolehan mereka untuk menghasilkan biofilem.Keputusan ini menunjukkan bahawa perbezaan kapasiti dalam penghasilan biofilem adalah disebabkan oleh perbezaan jenis protein permukaanA (spa) dan bukan disebabkan oleh perbezaan jenis MLST dan SCCmec.Pada bahagian ketiga kajian, kelaziman dan taburan molekul-molekul komponen matriks lekit permukaan mikrob (MSCRAMMs) dan gen-gen pada klon-klon berbeza *S. aureus* telah ditentukan. Kajian mendapati bahawa icaADBC, fnbA, eno, ebps, gen-gen clfA and clfB hadir dengan kelaziman yang tinggi dan sekata di antara berbagai-bagai klon jenis 60 MSSA and MRSA dari pencilan klinikal, Kelaziman gen-gen MSCRAMM pula adalah pelbagai. Pada bahagian keempat kajian, profil transkripsi gen-gen khusus staphylococcal yang mengekod MSCRAMMS and icaADBC telah ditentukan semasa perubahan kompleksiti penghasilan biofilem yang konsisten pada masa pertumbuhan yang berbeza.Keputusan menunjukkan bahawa ekspresi relatif gen-gen MSCRAMMS dan icaADBC

berbanding morfologi biofilem fenotipik boleh digunakan sebagai model untuk mengkaji regulasi naik- dan turun- gen-gen sedemikian.Ekspresi tertunda sesetengah gen semasa fasa pegun biofilem yang ditumbuhkan pada peringkat signifikan yang lebih tinggi telah dikenalpasti sebagai penting untuk pembangunan dan ikhtiar hidup bagi sel-sel pengkompos pada *niche* yang sukar untuk mendapatkan nutrien.Dalam bahagian kelima kajian ini, profil extracellular 2DE protein di kalangan genotypic klon yang berbeza di bawah perbezaan tempoh masa pertumbuhan biofilem oleh *S. aureus* telah dicirikan. Dalam kajian 2DE ini, keputusan yang paling utama dilihat adalah terdapat kepelbagaian extracellular protein di kalangan pelbagai klondan di bawah berbeza pertumbuhan masa-mata, menunjukan terdapat perbezaan pengawalan mod dalam proses pertumbuhan yang beroperasi di bawah jenis klon yang berbeza dan dalam situasi masa yang berbeza. Dalam bahagian keenam kajian ini, kepekaan ujian antibiotik (*glycopeptide, β-lactam, lipopeptide, oxazolidinones* dan *glycylcycline*) daripada jenis klon *S. aureus* ditentukan.Daripada kajian ini, didapati terdapat perbezaan yang agak besar dalam MIC dan aktiviti bakteriasidal daripada ejen-ejen kelas di kalangan jenis *spa* yang berbeza. Justeru itu, MIC dan MBC di kalangan klon yang sama jenis *spa* dan MLST didapati mempunyai perbezaan yang sedikit. Tambahan pula, *kepekatan pengurangan biofilm minimum.*daripada ejen-ejen ini dalam pencegahan formasi biofliem *in vitro* adalah agak tinggi secara keseluruhan berbanding dengan CLSI- ditakrifkan plaktonik MIC titik penentu untuk rintangan dan agak berbeza dikalangan jenis klon yang berbeza. Oleh demikian, kepelbagaian dalam kepekaan antibiotic daripada isolate dalam pelbagai jenis klon menekankan keperluan untuk pemantauan berterusan untuk klon dan doktor harus mempertimbangkan antibiotik yang betul dan bukannya rawatan empirikal. Dalam bahagian terakhir kajian ini, kesan kepekatan sub-perencat vancomycin dan tigecycline ke atas kadar

transkripsi mRNA MSCRAMM pada keadaan mantap dan target gen *ica*ADBC, dan ke atas rembesan eksoprotin daripada pelbagai klon pencilan S. aureus telah dikaji.Keputusan menunjukkan kesan antibiotik ini secara umumnya memberi kesan semua faktor kebisaan gen sasaran terpilih dan rembesan exoproteins. Oleh kerana ia mungkin meningkatkan penularan bakteri ini, penggunaan antibiotik untuk merawat jangkitan S. aureus mungkin menyebabkan keadaan yang lebih teruk.

kesimpulan: Kami menyimpulkan bahawa perbezaan yang besar wujud di kalangan jenis klon yang serupa dan pelbagai *S. aureus*.Perubahan ini boleh menyumbang kepada tahap kejahatan walaupun dalam genotip yang sama klon dan kepelbagaian dipertingkatkan dalam potensi jangkitan.Oleh itu kepelbagaian genetik baru menunjukkan bahawa pembangunan profil pengenalan pesat dan tepat bagi setiap jenis klon dalam jangkitan manusia adalah sangat penting untuk menetapkan antibiotik yang betul dan mengurangkan rawatan empirikal

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Last but not least, my deep apologizes for my mistakes during this time period and deep apology to all the peoples that a name didn't mentioned here.

I certify that a Thesis Examination Committee has met on 28.6.2013 to conduct the final examination of Salman Sahab Atshan on his thesis entitled" identification and characterization of biofilm-producing clinical isolates of *Staphylococcus aureus"* 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Rozita bt Rosli,PhD
Professor
Institute Bioscience
Universiti PutraMalaysia
(Chairman)

Fatimah bt Abu Baker, PhD
Professor
Faculty of Science and Food Technology
Universiti PutraMalaysia
(Internal Examiner)

Raha bt Hj Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Floyd C Knoop, PhD
Professor
Department of Medical Microbiology and Immunology
Creighton University School of Medicine
Omaha NE 66178
USA
(External Examiner)

_____
NORITAH OMAR, PhD
Assoc. Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

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

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

Rukman Awang Hamat, Master
Associate Professor
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)

Leslie Than Thian Lung, Phd
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, 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 citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

SALMAN SAHAB ATSHAN

Date: 28 June 2013

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