UNIVERSITI PUTRA MALAYSIA

ELUCIDATING PATHOGENIC DETERMINANTS IN STENOTROPHOMONAS MALTOPHILIA PATHOGENESIS

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By

RENJAN THOMAS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the requirements for the Degree of Doctoral of Philosophy

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Dedicated

In the name of

St. Gregorious Thirumeni of Parumala.

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

ELUCIDATING PATHOGENIC DETERMINANTS IN *STENOTROPHOMONAS MALTOPHILIA* PATHOGENESIS

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Chairperson: Associate Professor Vasanthakumari Neela, Ph.D

Faculty: Medicine and Health Sciences

Stenotrophomonas maltophilia, Gram negative bacteria has been known to be an environmental microbe with numerous biotechnological applications. They are ubiquitously found in nature. In recent times, this bacterium has been documented to be one of the leading nosocomial pathogen next to *Pseudomonas aeruginosa.* Owing to the high incident rate in hospital setup, they have been ranked as an opportunistic pathogen and have been associated with bacteremic infection and pneumonia, both with high rate of mortality in immunocompromised patients. Mortality rate has been found to be high with patients who have a history of prolonged hospitalization, malignancy, neutropenia, immune suppression, breakdown of muco-cutaneous defence barriers (e.g., following catheterization, artificial implantation, tracheotomy, or peritoneal dialysis), exposure to broad spectrum antibiotics and those requiring mechanical ventilation.

Their intrinsic/acquired resistance to most antibiotics and their ability to colonize surfaces of medical devices makes *S. maltophilia* a potentially dangerous pathogen. Treatment of *S. maltophilia* infections is also complicated by the fact that isolates are inherently resistant to many of the currently available broad-spectrum agent including carbapenems. Whether *S. maltophilia* clinical isolates are colonizers or true pathogens is still controversial.

Despite the increase in the spectrum of clinical syndromes associated with *S. maltophilia,* very little is known about the extracellular enzymes profiles which may have potential roles in pathogenesis especially among clinical isolates associated with infections. In this study, we screened and compared an array of extracellular enzymes in *S. maltophilia* collected from invasive and non-invasive clinical specimens by substrate plate assays. We also grouped the isolates as device related and non-device related and compared the enzyme profile. Our study showed all clinical isolates produced substantial levels of biochemical enzyme assayed. However, lecithinase and heparinase were significantly associated with isolates of invasive origin. In contrast, device related and non-device related did not show any

major significant difference. These data suggest that clinical isolates of *S. maltophila* are a reservoir for pathogenic potential enzymes.

The pathogenic potential of *S. maltophilia* strains isolated from clinical samples were screened for a panel of putative virulent genes such asputative lipase, putative iron complex outer membrane [ICOM], putative siderophore, *lux R, toxA, piliZ* and *tatD* which were fished out from closely related *P. aeruginosa* genome. The results showed that among the 108 isolates, 57.4%, 10.1%, 0.92%, 57.4% and 74% of the isolates harboured ICOM (n = 62), siderophore (n = 11), *luxR* (n = 1), Lipase (n = 62) and *tatD* (n = 80) harboured these genes. *ToxA* and *piliz* were not found in these clinical isolates. Relative quantification of these putative virulent genes showed ICOM, *tatD* and lipase genes to be overexpressed compared to others. Environmental strain *S. maltophilia* LMG 959 lacked these putative virulent genes.

The role of *S. maltophilia* on macrophages was studied to determine the inflammatory response and to study the phagocytic ability of this bacterium on RAW 264.7 macrophages. Both invasive and non-invasive isolates of *S. maltophilia* were able to enter the macrophage cells. Greater internalization ability was observed by clinical isolates of*S.maltophilia* in comparison to that of the environmental strain. *S. maltophilia* LMG959 ($p < 0.05$). Although all isolates of *S. maltophilia* gained entry, only the clinical isolates were able to replicate within the macrophages. Environmental strain was unable to replicate within the macrophage. The ability of clinical isolates of *S. maltophilia* to enter and survive the macrophages indicates its resistance to host defence system. Clinical isolates of *S. maltophilia* induced an amplified level of activation within macrophages which triggered immune response compared to environmental strains, as revealed by increased nitric oxide production and CD40 expression. Intracellular survivability of *S. maltophilia* was also ascertained by the presence of several bacteria which were observed as membrane bound. This intracellular phase during infection could play a prominent role in immune evasion and its pathogenicity.

In conclusion, *S. maltophilia* has all the essential qualities to be termed as a serious nosocomial pathogen with the presence of these virulence factors such as the extracellular enzymes and the gene products which could have a deleterious effect owing to the fact that the virulent determinants act in combination. Evading host defences and having intracellular survival ability makes this bacterium a potent and serious nosocomial pathogen.

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PENGENALPASTIAN PENENTU KEPATOGENAN YANG TERLIBAT DALAM PATOGENESIS *STENOTROPHOMONAS MALTOPHILIA*

Oleh

RENJAN THOMAS

2014

Pengerusi: Profesor Madya Vasanthakumari Neela, PhD

Fakulti: Perubatan dan Sains Kesihatan

Stenotrophomonas maltophilia, ialah bakteria negatif yang telah dikenalpasti sebagai mikrob persekitaran dengan pelbagai aplikasi-aplikasi bioteknologi. Ia boleh didapati di mana-mana sahaja di dalam persekitaran. Pada masa kini, bakterium ini telah didokumentasikan sebagai salah satu patogen nosokomial selepas *Pseudomonas aeruginosa*. Berdasarkan kepada kadar cerapan yang tinggi di dalam hospital, *S. maltophilia* telah diklasifikasikan sebagai patogen pengambil peluang dan telah dikaitkan dengan bakteremia dan pneumonia, kedua-duanya dengan kadar mortiliti yang tinggi terutama sekali di kalangan pesakit-pesakit yang tercabar sistem imuniti mereka. Kadar mortiliti adalah tinggi di kalangan pesakit-pesakit dengan rekod penghospitalan yang berpanjangan, barah, nutropenia, pembantutan sistem imuniti, kerapuhan pada batas pertahanan-pertahanan mukus-kulit (cth., berikutan pada prosedur kateter, pengimplanan palsu, trakeotomi atau dialisis peritoneal), pendedahan kepada antibiotik spektrum lebar dan mereka-mereka yang memerlukan ventilasi mekanik. Rintangan secara dalaman dan yang diperolehi pada kebanyakan antibiotik – antibiotik dan kepada kebolehan mereka untuk mengkolonisasi permukaan – permukaan peralatan perubatan menjadikan *S. maltophilia* sebagai patogen yang berpotensi berada pada tahap merbahaya. Sama ada *S. maltophilia* dipencilkan secara klinikal merupakan pengkolonisasi atau patogen benar masih menjadi tanda persoalan.

Meskipun terdapat peningkatan di dalam spektrum yang melibatkan sindrom klinikal dengan *S. maltophilia*, amat sedikit pengetahuan tentang profil – profil enzim luar sel yang berkemungkinan mempunyai potensi di dalam penglibatannya dalam patogenesis terutama sekali pemencilan yang diperolehi daripada jangkitan-jangkitan yang berhubung kait dengan kes klinikal. Di dalam kajian ini, satu siri pelbagai enzim-enzim luar sel yang terdapat pada *S. maltophilia* yang dikumpulkan daripada spesimen klinikal invasif dan bukan invasif disaringkan dengan asai 'substrate plate'. Semua pemencilan klinikal menghasilkan tahap – tahap asai biokimia enzim yang memberangsangkan. Walau bagaimanapun, enzim lesitinase dan heparinase memainkan peranan yang penting di dalam pemencilan secara invasif. Apabila pemencilan – pemencilan ini diklasifikasikan sebagai berkaitan dengan peralatan dan bukan berkaitan dengan peralatan, ia tidak menunjukkan

sebarang perbezaan signifikasi yang major di dalam profil-profil enzim. Kesemua data ini mencadangkan bahawa pemencilan secara klinikal akan *S. maltophilia* adalah lombong untuk mendapatkan enzim – enzim yang mempunyai potensi untuk menunjukkan ciri-ciri patogenik.

Potensi patogenik strain – strain *S. maltophilia* yang dipencilkan daripada sampel-sampel klinikal disaringkan pada satu panel gen-gen virulansi putatif seperti lipase, kompleks besi luar membran (ICOM), siderofor, *lux R, toxA, pili Z* dan *tat D* yang mana dikail keluar daripada genom *P. aeruginosa* yang berkait rapat. Keputusan – keputusan amali menunjukkan bahawa di antara 108 pencilan, 57.4 %, 10.1%, 0.92%, 57.4% dan 74% pencilan – pencilan ini mempamerkan gen –gen ICOM (n = 62), siderofor (n = 11), *lux R* (n = 1), lipase (n = 62) dan*tatD* (n = 80). *ToxA* dan *piliz* tidak terdapat di dalam pencilan – pencilan klinikal ini. Kuantifikasi relatif akan gen-gen virulansi putatif ini menunjukkan gen – gen ICOM, *tatD* dan lipase diekspresikan secara berlebihan berbanding gen – gen yang lain. Strain persekitaran *S. maltophilia* LMG 959 tidak mempunyai gen-gen virulansi putatif ini.

Peranan *S. maltophilia* ke atas makrofaj – makrofaj dikaji untuk menentukan responsi keradangan dan untuk mengkaji kebolehan patogenik bakterium ini ke atas makrofaj - makrofaj RAW 264.7. Kedua – dua pencilan invasif dan bukan invasif bakteria *S. maltophilia* telah menunjukkan kebolehannya untuk memasuki dalam sel-sel makrofaj. Pencilan – pencilan klinikal menunjukkan kebolehan untuk duduk di dalam sel berbanding dengan pencilan persekitaran *S. maltophilia* LMG959 yang tidak berjaya melakukan yang demikian ($p < 0.05$). Walaupun semua pencilan – pencilan *S. maltophilia* telah berjaya memasuki sel – sel makrofaj, hanya pencilan klinikal sahaja yang berkebolehan untuk mengembangbiak di dalam makrofaj – makrofaj tersebut. Strain persekitaran tidak berjaya untuk mengembangbiak di dalam makrofaj. Kebolehan pencilan - pencilan klinikal *S. maltophilia* untuk memasuki dan hidup di dalam makrofaj- makrofaj memberi implikasi bahawa ia adalah rintang kepada sistem pertahan tubuh induk. Pencilan – pencilan klinikal *S. maltophilia* mampu untuk mengaruh tahap amplifikasi pengaktifan gen - gen di dalam makrofaj – makrofaj berbanding dengan strain – strain persekitaran, sebagaimana yang disahkan dengan peningkatan nitrik oksida (NO) dan ekspresi CD40. Kebolehan untuk hidup di dalam sel oleh *S. maltophilia* juga telah dipastikan dengan kehadiran beberapa bakteria di mana permerhatiannya menunjukkan ianya adalah berikatan dengan membran sel. Fasa dalam sel semasa jangkitan ini mungkin memainkan peranan yang penting di dalam pencerobohan imun dan kepatogenisitiannya.

Kesimpulannya, *S. maltophilia* mempunyai semua kualiti yang diperlukan untuk diklasifikasikan sebagai patogen nosokomial yang serius dengan kehadiran faktor-faktor virulansi putatif seperti enzim-enzim luar sel dan produk – produk gen di mana ia berkemungkinan mempunyai kesan – kesan yang boleh merosakkan apatah lagi diketahui bahawa penentu virulansi berkesan dalam bentuk kombinasi. Mengelak sistem pertahan induk dan mempunyai kebolehan hidup di dalam sel induk menjadikan bakterium ini patogen nosokomial yang serius dan kuat. Maka, kajian yang lanjut perlu dijalankan bagi memahami fungsi faktor-faktor virulansi dan mekanisma patogenesis bakteria ini.

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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:

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DECLARATION

Declaration by graduate student

I hereby confirm that:

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Declaration by Members of Supervisory Committee

This is to conform that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2002 (Revision 2012-2013) are adhered to.

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AM	Alveolar macrophages
AMP	Adenosine monophosphate
APC	Antigen presenting cells
ATCC	American type culture collection
BCCM	Belgian Co-ordinated Collections of Microorganisms
BEC	Bladder epithelial cells
BSA	Bovine serum albumin
CA	Community acquired
CD40	Cluster of differentiation 40
CF	Cystic fibrosis
CRP	Cyclic AMP receptor protein
CSF	Cerebrospinal fluid
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DSF	Diffusible signal factor
EDTA	Ethylene diaminetetra acetic acid
EPS	Exopolysaccharide
FACS	Fluorescence activated cell sorting
HA	Hospital acquired
HCA	Healthcare associated
HIV	Human immunodeficiency virus
ICOM	Iron complex outer membrane protein
IFA	Immunofluorescence assay

IL10	Interleukin 10
IL-1β	Interleukin -1beta
IL8	Interleukin 8
iNOS	inducible nitric oxide synthase
LB	Luria bertani
LPS	Lipopolysaccharide
MAMP	Microbe associated molecular patterns
MDR	Multiple drug resistant
MHA	Muller hinton agar
MOI	Multiplicity of infection
NED	N-1- napthyl ethylenediamine Dihydrochloride
NET	Neutrophil extracellular traps
NGM	Nematode growth hormone
NO	Nitric oxide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PL	Polysaccharide lyase
PRR	Pattern recognition receptors
QS	Quorum sensing
rRNA	Ribosomal ribonucleic acid
RTI	Respiratory tract infection
SMF-1	*S. maltophilia* fimbriae 1
SS-PCR	Species specific polymerase

	chain reaction
T2S	Type II secretion system
TEM	Transmission electron microscopy
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Stenotrophomonas maltophilia is an emerging nosocomial, Gram-negative and multiple-drug-resistant (MDR) pathogen. This non-fermenting, bacilli has underwent numerous taxonomic changes over the time (Palleroni and Bradbury 1993). This bacterium has been associated with serious infections in humans (Sader, Jones et al. 2005; Crossman, Gould et al. 2008) and widely known to be an important nosocomial pathogen in immunosuppressed patients (Almeida, Rubio et al. 2007).

Ubiquitous in nature, *S. maltophilia* are found in a variety of microenvironments and geographical regions and occupies distinct ecological niches such as water, vegetables and soil. Owing to the fact that *S. maltophilia* are environmental bacteria and they inherit the multiple-drug-resistant (MDR) property, these microbes have also been isolated from aqueous sources within and outside the clinical setup. In the environment, it has been isolated from soil (Minkwitz and Berg 2001), plant roots (Berg 2009), animals (Hejnar, Kolář et al. 2010), and invertebrates (Petridou, Filioussis et al. 2010). In hospitals, isolation of *S. maltophilia* has been reported from hospital instruments such as ventilators (Kollef, Silver et al. 1995), central venous catheters (Muder, Harris et al. 1996), arterial pressure monitors, dialysis equipment (Flaherty, Garcia-Houchins et al. 1993), endoscopes (Kovaleva, Degener et al. 2010), hospital suction tubing (Yorioka, Oie et al. 2010), ice machines (Denton and Kerr 1998), tap water (Cervia, Farber et al. 2010), sinks (Brooke, Vo et al. 2008) and disinfectants (Mukhopadhyay, Bhargava et al. 2003). They have also been isolated from surfaces of materials used in intravenous cannulae, prosthetic devices and nebulizers (Denton, Rajgopal et al. 2003).

Although not a primary pathogen, *S. maltophilia* has emerged as an opportunistic nosocomial (hospital-acquired) microorganism. Most commonly *S. maltophilia* infections are associated with respiratory tract infections like pneumonia (Sefcick, Tait et al. 1999) and acute exacerbations of chronic obstructive pulmonary disease [COPD] (Nseir, Di Pompeo et al. 2006), bacteremia (Lai, Chi et al. 2004), biliary sepsis (Papadakis, Vartivarian et al. 1995), infections of the bones and joints, urinary tract, soft tissues (Sakhnini, Weissmann et al. 2002; Landrum, Conger et al. 2005; Bin Abdulhak, Zimmerman et al. 2009), endophthalmitis (Akçakaya, Sargın et al. 2011), eye infections, keratitis, scleritis, dacryocystitis (Mauger, Kuennen et al. 2010; Lin, Ma et al. 2011; Wladis 2011), endocarditis (Takigawa, Noda et al. 2007) and meningitis (Rojas, Garcia et al. 2009). Hospital-acquired infection of *S. maltophilia* has been increasing among immunocompromised population with high rates of mortality ranging from 20 to 70% (Farrell, Sader et al. 2010). Prolonged hospitalization, chemoprophylaxis, heart surgery (Del Toro, Rodriguez-Bano et al. 2006; Paez and Costa 2008) and burns (Tsai, Chen et al. 2006) are major risk factors involved in *S. maltophilia* colonization/infection. High rates of isolation in immunocompromised patients, increasing multidrug resistant strains, and lack of controlled clinical treatment trials makes this bacterium a cause of serious concern (Rolston, Kontoyiannis et al. 2005; Nicodemo and Paez 2007; Safdar and Rolston 2007).

Whether *S. maltophilia* is a colonizer or true pathogen is still unanswered and critical as isolation of pure *S. maltophilia* from lungs of pneumonic patients is difficult (Pathmanathan and Waterer 2005). Production of several extracellular enzymes that includes DNase, RNase, fibrinolysin, lipases, lecithinases, hyaluronidases, proteases and elastases associated with virulence has been reported in *S. maltophilia* infection and pathogenesis (Denton and Kerr 1998; Crossman, Gould et al. 2008). Adherence to biotic and abiotic surfaces, biofilm forming ability, antibiotic resistance to a whole wide group of antibiotics, presence of outer- membrane lipopolysaccharide and resistance to complement mediated killing are other properties that qualifies *S. maltophilia* as a pathogen to initiate and establish infection (Looney, Narita et al. 2009). Besides, studies have shown that *S. maltophilia* has immunostimulatory property to induce tumor necrosis factor alpha (TNF-α) which contributes significantly to airway inflammation (Waters, Gomez et al. 2007), the actual information pertaining to the immune response between host and this bacteria is still lacking. Regardless the association of *S. maltophilia* with several serious illnesses and presence of virulence factors that aids in damaging the host tissues making the host permissive for infection the pathogenicity potential or virulence property is not clearly understood.

In conclusion there is an immediate need to study and characterize the importance of virulence factors involved in the pathogenesis of *S. maltophilia* which is emerging as a significant nosocomial pathogen. In this context, the present study employing clinical isolates of *S. maltophilia* was undertaken to identify and screen isolates for their ability to produce different virulence factors or gene products. Their capability to elicit an immune response was studied.

The specific objectives pertaining to this study are as follows:

1. To determine the extracellular enzyme profiling of *S. maltophilia* isolated from clinical samples.
2. To investigate the prevalence of putative virulent genes in *S. maltophilia* infection.
3. To study the intracellular ability of *S. maltophilia* on RAW 264.7 macrophages and immune response *in vitro*.

Thesis Organization

This study was organized into 6 chapters. The chapters are formatted according to the style 2 of the Guide to Thesis Preparation April 2009, School Of Graduate Studies, Universiti Putra Malaysia. Chapters 1 and 2 are identified as introduction and literature review, respectively. Chapters 3 to 5 are identified as research chapters and study stands on its own. Chapter 6 is identified as summary, conclusions and recommendations.

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