

**DEMONSTRATION OF ANTIGENIC AND SPECIFIC OUTER MEMBRANE
PROTEIN(S) OF *Acinetobacter baumannii***

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**DEMONSTRATION OF ANTIGENIC AND SPECIFIC OUTER MEMBRANE
PROTEIN(S) OF *Acinetobacter baumannii***

by

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**Thesis submitted in fulfillment of the
requirements for the degree
of Master of Science**

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LIST OF ABBREVIATIONS & SYMBOLS

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AP	Alkaline Phosphatase
ATCC	American Type Culture Collection
BCCM	Belgian Co-ordinated Collections of Microorganisms
DNase	Deoxyribonuclease
EIA	Enzyme <u>Immuneassay</u> <u>Immunoassay</u>
ELISA	Enzyme Linked Immunosorbent Assay
HRP	Horse Radish Peroxidase
HUSM	Hospital Universiti Sains Malaysia
ICT	Immunochromatography
ICU	Intensive Care Unit
IDSA	Infectious Disease Society of America
IMP	Inner Membrane Protein
kDa	Kilodalton
mA	Milliampere
MDR	Multi Drug Resistant
MDRAB	Multi Drug Resistant <i>Acinetobacter baumannii</i>
MW	Molecular weight
NC	Nitrocellulose Membrane
OD	Optical Density
OMP	Outer Membrane Protein
OXA	Oxacillinase
PAI	Pathogenicity Island
PBS	Phosphate Buffered Saline
PPSP	Pusat Pengajian Sains Perubatan
RNase	Ribonuclease
SAP	Surface Associated Protein
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
USD	United States Dollar
USM	Universiti Sains Malaysia
WHO	World Health Organization
°C	Degree Celcius
µg	Microgram
mg	Milligram
g	Gram
nM	Nanometer
A ₂₈₀	Absorbance at 280 nM

DEMONSTRASI KEHADIRAN PROTEIN MEMBRAN LUAR YANG ANTIGENIK DAN SPESIFIK BAGI *Acinetobacter baumannii*

ABSTRAK

Acinetobacter baumannii dikenali sebagai bakteria penyebab penyakit nosokomial dan kebanyakannya adalah rintang terhadap pelbagai antibiotik. Ianya juga dikenalpasti sebagai penyebab utama kepada morbiditi dan kematian di hospital terutamanya bagi pesakit yang kurang imuniti terhadap penyakit. Diagnosis awal bagi jangkitan yang disebabkan oleh *A. baumannii* adalah strategi penting untuk mengawal jangkitan nosokomial yang disebabkan oleh bakteria ini. Pengenalpastian bakteria ini pada masa kini adalah dengan menggunakan kaedah pengkulturan konvensional dan ujian biokimia yang mengambil masa lebih kurang 2 hingga 7 hari. Oleh sebab itu, ujian yang cepat, sensitif, spesifik dan murah diperlukan untuk pengurusan yang cepat terhadap jangkitan nosokomial ini.

Pembangunan ujian yang spesifik dan sensitif memerlukan *biomarker* yang tidak bertindakbalas silang dengan bakteria lain dan spesifik hanya untuk *A. baumannii*. Oleh itu, tujuan kajian ini dilakukan adalah untuk mengenalpasti kehadiran protein yang spesifik dan antigenik terhadap *A. baumannii* daripada protein membran luar (OMP) yang boleh digunakan untuk membangunkan ujian diagnostik yang cepat dan spesifik. Profil protein daripada strain ATCC and isolat klinikal *A. baumanii* telah didemonstrasi dengan menggunakan teknik SDS-PAGE dan profil protein yang terhasil daripada kedua-dua strain tersebut dibandingkan. Profil protein daripada isolat klinikal didapati mempunyai persamaan lebih kurang 90% dengan strain ATCC. Seterusnya, protein elektroforetogram tersebut diuji dengan analisis blot

Western yang ditindak balas dengan serum daripada pesakit yang dijangkiti dengan *A. baumannii*. Satu protein OMP yang antigenik dan juga spesifik berberat molekul 34.4 kDa telah dapat dikenalpasti hadir pada kesemua isolat *A. baumannii* yang dikaji dan tidak bertindak balas silang dengan serum daripada pesakit yang dijangkiti dengan pathogen nosokomial lain atau kontrol normal yang diuji. Eksperimen tersebut diulang beberapa kali untuk mengesahkan keputusan tersebut.

Kajian ini juga dijalankan untuk menilai kesan peningkatan suhu terhadap pengekspresan protein OMP. Profil protein pada suhu 41°C menunjukkan sebilangan OMP diekspres dengan kuantiti lebih tinggi (17.3, 22.4 and 60.5 kDa), sementara sebahagian protein yang lain menunjukkan penurunan dalam kuantiti protein yang diekspresi. Keputusan ini mengesyorkan bahawa peningkatan suhu badan semasa jangkitan *A. baumannii* mempengaruhi pengekspresan protein bakteria tersebut, kemungkinan sebagai mekanisma pertahanan terhadap suhu tinggi dan juga rintangan terhadap dadah/ubat bagi memastikan bakteria tersebut boleh hidup dan tumbuh dalam badan pesakit.

Keputusan ujian ini juga menunjukkan bahawa protein 34.4 kDa tersebut hadir pada kedua-dua penyediaan OMP dan SAP daripada *A. baumannii* serta bukan sejenis glikoprotein. Antigen 34.4 kDa hadir dalam ke semua isolat klinikal yang diuji dan didapati ia diekspres dengan kuantiti lebih tinggi pada suhu 41°C. Ini mencadangkan bahawa protein ini mempunyai peranan yang penting dalam mekanisma patogenisiti bakteria tersebut. Sehingga kini, tiada kajian yang dilaporkan mengenai protein yang spesifik terhadap *A. baumannii*. Keputusan kajian yang diperolehi adalah memberansangkan dengan penemuan protein 34.4 kDa yang spesifik terhadap *A.*

baumannii dan boleh digunakan sebagai *biomarker* dalam pembangunan ujian diagnostik yang lebih cepat dan lebih spesifik jika dibandingkan teknik ujian diagnostik yang digunapakai pada masa kini. Walau bagaimanapun, kajian lanjutan perlu dilakukan untuk mengukur tahap antibodi terhadap protein tersebut, sensitiviti dan spesifisiti dan tempoh masa antibodi terhadap protein tersebut dapat dikesan di dalam serum pesakit.

DEMONSTRATION OF ANTIGENIC AND SPECIFIC ANTIGENIC AND SPECIFIC OUTER MEMBRANE PROTEIN(S) (OMPs) OF *Acinetobacter* *baumannii*

ABSTRACT

Acinetobacter baumannii has been recognized as an emerging nosocomial pathogen and is very often multi-resistant to antibiotics. It has also been identified as an important cause of morbidity and mortality in hospitals, especially among immunocompromised patients. Early diagnosis of infection caused by *A. baumannii* is the major strategy for limiting controlling the nosocomial infection caused by this pathogen. Current identification of this of the bacteria is by conventional culture method and biochemical tests, which may take s about 2 to 7 days to produce results. Hence, there is a need for a new rapid, sensitive, specific and economical test that would allow for the rapid management of nosocomial *A. baumannii* infections.

Development of a specific and sensitive diagnostic test requires a biomarker, which does not cross react with other bacteria and is specific only to *A. baumannii*. This formed the aim of this study; to detect the presence of a specific and antigenic biomarker for *A. baumannii* from the outer membrane proteins (OMPs), which can be used for the development of a rapid and specific diagnostic test. —Protein profiles of OMP lysates from the ATCC strain —(Belgium) and clinical isolates of *A. baumannii* (Department of Medical Microbiology and Parasitology, School of Medical Sciences, USM) were

obtained by applying demonstrated using the technique of Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and the protein profiles were compared. The protein profiles of the clinical isolates were 90% identical to that of the ATCC strain. Following this, the protein electrophoretograms were subjected to Western blot analysis using serum from patients infected with *A. baumannii* and non-*A. baumannii*. The Western blot analysis revealed a 34.4 kDa antigen which was immunogenic when probed IgA, IgM and IgG of *A. baumannii* sera but did not cross reacted with sera from other nosocomial infections and normal controls. This was confirmed by repeated testing.

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The 2 sera (AB 009 and AB 012) from *A. baumannii* infection showed 19 and 16 positive bands respectively of which 4 bands were recognized by both the sera. These 4 protein bands were checked for cross reactivity using sera from patients infected with *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* or normal controls. The three proteins other than the 34.4 kDa protein cross reacted with sera from other nosocomial infections or normal controls. The 34.4 kDa antigen did not show any cross reaction with sera from other nosocomial infections and normal controls suggesting that this protein was specific for *A. baumannii*. This was confirmed by repeated testing.

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Studies were also done to :-

(i) assess the effect of temperature (41°C identical to the temperature in patients with fever during nosocomial infection) on the expression of the OMPs. (ii) determine the location of the protein by SDS-PAGE analysis of the OMPs. Surface associated proteins

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(SAPs) and inner membrane proteins (IMPs) of *A. baumannii* in order to identify the location of the protein.

The proteinOMPs profile expressed at 41°C showed a number of few proteins were over-expressed, OMPs to be increased in expression (17.3, 22.4 and 60.5 kDa) while some proteins also showed were down-regulated ion suggesting that the higher elevated body temperature of the body during *A. baumannii* infection influences the expression of the bacterial proteins for survival of this bacterium, probably as a mechanism of survival at higher temperatures and also for resistance against drugs to ensure its survival and growth in the body.

The 34.4 kDa antigen, was present in all the clinical isolates and hence can be considered as a specific biomarker with great potential as a diagnostic marker. However, the effect of temperature was not uniform in the clinical isolates studied showing increased expression of this protein in 60% of isolates and the down regulation or no effect in 40% of the clinical isolates. This suggests that this protein may not have a strong protective role to play and hence may not be suitable as a vaccine candidate.

It was found that Further characterization of the 34.4 kDa protein demonstrated that it was associated with both OMPs and SAPs of *A. baumannii* and -is. Besides this, the protein was also analysed for its glycosylation status using glycoprotein staining and also for the main constituents using trypsin digestion. Results showed that it was not a glycoprotein, its main constituents being protein. The 34.4 kDa antigen was present in all the clinical isolates of *A. baumannii*. The expression of this protein was enhanced in

most cases suggesting that this protein could also be related to the virulence of the bacteria. To date, no previous report is available with reference to this specific protein for *A. baumannii*.

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Overall, the results of this study has identified a unique protein expressed by the *A. baumannii* clinical isolates which is specific to *A. baumannii* and does not cross react with other bacterial species responsible for nosocomial infections. Western blot analysis showed the protein to be antigenic and induce antibodies. Chemical characterization showed that its main constituent is protein and is not glycosylated.

The results are encouraging in that the 34.4 kDa protein identified is specific for *A. baumannii* and can be used as a biomarker for development of a diagnostic test which would be faster and more specific than the current techniques of diagnosis. However, further studies need to be done to measure the antibody level against this specific protein, the sensitivity and specificity of the protein and the retention time of the antibody detectable in the serum of the infected patients. Since routine culture methods to identify the bacterial infection are laborious, time consuming, relatively expensive and low sensitivity, the development of a more rapid and simplified diagnostic test of *Acinetobacter* infection is highly desirable. The test must be sensitive, specific, and easy to perform, cost effective and be able to detect the presence of *A. baumannii* directly from patients' blood. As such the objective of the study was to determine the presence of a specific and antigenic outer membrane protein (OMPs) of *A. baumannii*, which can be used for the development of a rapid diagnostic test. Proteins profiles were obtained by

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applying the techniques of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analysis was done to detect the presence of IgM, IgA and IgG. All sera from patients infected with *A. baumannii* were collected from the Department of Medical Microbiology & Parasitology, School of Medical Sciences, USM. By the method of elimination, antigenic protein band with a molecular weight of 34.4 kDa, which, was uniquely seen only by *A. baumannii* sera and do not cross react with other sera tested was identified. This protein was shown to be antigenic when probed with anti-human IgM, IgA and IgG by using patients sera infected with *A. baumannii*. Moreover, it was found to be specific for *A. baumannii* and did not cross react with other sera that causing nosocomial infections (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* etc), most commonly found in hospital Universiti Sains Malaysia (HUSM). Study was also done to determine the location of the protein as to whether it is present on outer membrane only or present in other membranes (surface or inner membrane). It was found that this band was exist in surface associated protein. Besides this, the protein was also analysed for its glycosylation status using glycoprotein staining and also for the main constituents using trypsin digestion. Results showed that it is not a glycoprotein, its main constituents were protein. To date, no previous report has been made regarding the protein. However, further studies

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CHAPTER ONE

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INTRODUCTION

1.0 Introduction

Acinetobacter baumannii is a Gram-negative, non-motile, obligate aerobic ~~eeceus~~
~~coccobacilli~~, that is commonly found in soil, water and sewage, and in healthcare settings
(Baumann *et al.* 1968; Juni 1978 Dijkshoorn *et al.*, 2007; Perez *et al.*, 2007; Shih *et al.*,
2008). Difficulties in containing, controlling and eliminating the spread of *A. baumannii*
~~have~~are challenges faced by ~~challended~~ clinicians and healthcare providers (Bergogne-
Berezin and Towner 1996; Bernards *et al.* 2004; Koulenti and Rello, 2006). *A.
baumannii* has emerged as an important and problematic human pathogen as it is the
causative agent of several types of infections including pneumonia, meningitis,
septicaemia and urinary tract infections. Recently, ~~a~~ drug-resistant *A. baumannii* was
responsible for an outbreak of bacteraemia in more than 240 American troops in Iraq
(Centers for Disease Control and Prevention 2004; Abbott Davis *et al.*, 2005; Scott *et
al.*, 2007), and there is significant concern of ~~a~~ major epidemic involving this organism.
This versatile organism can utilize a variety of carbon sources and is able to grow in a
~~wide~~ range of temperatures (28-53°C) and pH conditions (Yavankar *et al.*, 2007). La
Scola and Raoult (2004) isolated *A. baumannii* from human body lice and speculated
that the bacteria may utilize the arthropod host as ~~a one~~ means of transmission. This
hardiness, combined with its intrinsic resistance to many antimicrobial agents,
contributes to the organism's fitness and has enabled it to thrive in hospital settings

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worldwide. Mortality in patients suffering from *A. baumannii* infections can be as high as 75% (Chastre and Trouillet, 2000).

Alarmingly, little is known about the virulence, antibiotic resistance, or persistent strategies of *A. baumannii*. The pathogenic determinants that have been reported thus far for *A. baumannii* include a novel pilus assembly system involved in biofilm formation (Tomaras et al. 2003), an outer membrane protein (Omp38) that causes apoptosis in human epithelial cells (Choi et al. 2005), and a polycistronic siderophore-mediated iron-acquisition system conserved between *A. baumannii* and *Vibrio anguillarum* (Dorsey et al. 2003, 2004). This presumably comprises a small fraction of elements involved in *A. baumannii* pathogenesis, and thus, novel global approaches are essential to comprehensively understand the basic features of this organism in order to ultimately control the spread of *A. baumannii* infections and to develop effective counter measures against this harmful pathogen

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~~control the spread of *A. baumannii* infections and to develop effective countermeasures against this harmful pathogen.~~

A. baumannii has been stealthily gaining ground as an agent of serious nosocomial and community-acquired infection. Historically, ~~Acinetobacter~~ spp. have been associated with opportunistic infections that were rare and of modest severity; the last two decades have seen an increase in both the incidence and seriousness of *A. baumannii* infection, with the main targets being patients in intensive-care units. Although this organism appears to have a predilection for the most vulnerable patients, community-acquired *A. baumannii* infection is an increasing cause for concern ([Chastre et al. 2000](#)). The increase in *A. baumannii* infections has paralleled the alarming development of resistance it has demonstrated. The persistence of this organism in healthcare facilities, its inherent hardiness and its resistance to antibiotics results in it being a formidable emerging pathogen.

1.1 History and significance of *Acinetobacter baumannii* infection

1.1.1 Epidemiology

A. baumannii ~~Acinetobacter baumannii~~ has emerged worldwide as an important nosocomial pathogen, causing outbreaks particularly in intensive care units, in wards with patients who have serious underlying illness (Dijkshoorn *et al.*, 2007). It is responsible for 2% ~~to~~–10% of all Gram-negative bacterial infections in intensive care units in Europe and the United States (Herve Richet and Pierre Edouard Fournier, 2006).

~~Imipenem is among the drugs of choice for treatment of nosocomial infections due to multidrug resistant (MDR) *A. baumannii* isolates. However, their efficacy is being~~

~~increasingly compromised by the emergence of carbapenem hydrolyzing β -lactamases of molecular Ambler class B (VIM, IMP) and class D (OXA-23, OXA-58) (Poirel et al., 2005; Coelho et al., 2006; Zong et al., 2008).~~

A. baumannii has emerged as a highly troublesome pathogen for many institutions globally. Multi-drug resistant *A. baumannii* ~~A. baumannii~~ The organism (MDRAB) has always been inherently resistant to multiple antibiotics. Multi-drug resistant *A. baumannii* is abbreviated as MDRAB. Imipenem is among the drugs of choice for treatment of nosocomial infections due to MDRAB strains. However, their efficacy is being increasingly compromised by the emergence of carbapenem-hydrolyzing β -lactamases of molecular Ambler class B (VIM, IMP) and class D (OXA-23, OXA-58) (Poirel et al., 2005; Coelho et al., 2006; Zong et al., 2008). As a consequence of its immense ability to acquire or up-regulate antibiotic drug resistance determinants, it has justifiably been propelled to the forefront of scientific attention. Apart from its predilection for the seriously ill within intensive care units, *A. baumannii* has more recently caused a range of infectious syndromes in military personnel injured in the Iraq and Afghanistan conflicts (described earlier Scott et al., 2007).

~~In conclusion, The available evidence suggests that *A. baumannii* is an important human pathogen that is gradually gaining more attention as a public health threat. It~~

causes a significant proportion of infections in specific patient populations, especially in critically-ill patients receiving care in the ICU setting (Zaragoza *et al.*, 2003; Lee *et al.*, 2004; Longo *et al.*, 2007). This situation, together with the fact that *A. baumannii* isolates have inherent and/or easily acquired mechanisms of resistance against many of the available antimicrobial agents, makes this pathogen one of the most significant microbial challenges of the current era. More scientific efforts and resources are urgently needed to further elucidate the epidemiological and infection control issues related to *A. baumannii* infections, and to investigate treatment options for patients with multidrug- or pandrug-resistant infections.

1.1.2 Classification/~~T~~axonomy

In 1980s, *Acinetobacter* was first considered as an emergence of nosocomial pathogens. Members of the genus *Acinetobacter* have a long story of taxonomic change. This confusion makes it difficult to interpret the older medical and scientific literature (Bergogne-Berezin and Towner, 1996). Bergey's Manual of Systematic Bacteriology classified the genus *Acinetobacter* in the family *Neisseriaceae*, but ~~this arrangement has never been formally approved by the taxonomists~~the taxonomists have never formally approved this arrangement. After that, taxonomic developments have resulted in the proposal that members of the genus should be classified in the new family *Moraxellaceae*. This genus *Acinetobacter*, ~~r~~r which is now defined as Gram negative (but sometimes difficult to destain) coccobacilli, with a DNA G ~~and~~+C content of 39 to 47 mol%, ~~that~~ are strictly aerobic, non-motile, catalase-positive, and oxidase-negative

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(Bergogne-Berezin and Towner, 1996). So far 17-named species have been recognized and 15 genomic species (gen.sp.) have been delineated by DNA–DNA hybridization, which do not yet have valid names (Dijkshoorn *et al.*, 2007).

1.1.3 Properties of *Acinetobacter baumannii*

1.1.3.1 Physical characteristics

Acinetobacter baumannii does not have fastidious growth requirements and is able to grow at various temperatures and pH conditions (Bergogne-Berezin *et al.*, 1996). The versatile organism exploits a variety of both carbon and energy sources. These properties explain the ability of *Acinetobacter* species to persist in either moist or dry conditions in the hospital environment, thereby contributing to transmission (Smith *et al.*, 2007). This hardiness, combined with its intrinsic resistance to many antimicrobial agents, contributes to the organism's yfitness-irulence and has enabled it to spread in the hospital setting (Abbo *et al.*, 2005). Clinical isolates of *A. baumannii* are capable of activating N-acylhomoserine-lactone biosensors with maximal activity in the stationary growth phase (Joly-Guillou *et al.*, 2005). *Acinetobacters* are renowned for their ability to survive in the environment in dry conditions for prolonged periods, and environmental contamination represents an important reservoir for their dissemination (Aygun-G-*et al.*, 2002). Another interesting feature of the catabolic capacity of *A. baumannii* is its inability to catabolize glucose. Recent report showed that this deficiency in *A. baumannii* as-is due to the absence of hexokinase, glucokinase, or any other comparable enzyme that can transfer phosphate onto glucose. Thus, the first step of glycolysis

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cannot be completed (Smith *et al.*, 200⁷⁵).

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1.1.3.2 Growth and cultural characteristics

Acinetobacter grows rapidly on 5% sheep blood and MacConkey agars. Characteristic of colonies on 5% sheep blood agar producing smooth, opaque colonies, in which some isolates are β-haemolytic. Colonies on MacConkey agar are light lavender colour indicating but do not ferment lactose.

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1.1.3.3 Biochemical characteristics

Acinetobacter baumannii is a oxidase-negative, catalase-positive and urease-positive bacterium. It shows no reaction with indole and methyl red. In the Triple Sugar Reaction-Iron (TSI) agar, it shows alkaline slant and neutral butt and it does not produce gas (H_2S).

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1.1.3.4 Physiology and Morphology

Members of the genus *Acinetobacter* are non-motile coccobacilli that are frequently confused with *Neisseriae* in Gram stained samples. They are generally encapsulated, **oxidase negative, catalase positive, obligate aerobic** and they do not ferment carbohydrates. *Acinetobacter* spp. are short, plump, Gram negative (but sometimes difficult to destain) rods, typically 1.0 to 1.5 μm by 1.5 to 2.5 μm in **size during** the logarithmic phase of growth but often becoming more coccoid in the stationary phase (Bergogne-Berezin and Towner, 1996).

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1.2 Clinical significance of *Acinetobacter baumannii*

Acinetobacter-*A.* *baumannii* is an important nosocomial pathogen that has been implicated in various **ranges of** infections that mainly affect critically ill patients in ICUs. Hospital-acquired infections caused by *A. baumannii* includes bloodstream infections, ventilator-associated pneumonia, skin and soft-tissue infections, wound infections, respiratory and urinary-tract infections, endocarditis, secondary meningitis **etc and other infections-** (Joly-Guillou *et al.*, 2005; Lee *et al.*, 2006; Dijkshoorn *et al.*, 2007; Lee *et al.*, 2008). **These infections are mainly attributed to *A. baumannii*, although gen.sp. 3 and gen.sp. 13TU have also been implicated (Dijkshoorn *et al.*, 2007).**

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Nosocomial infections that are caused by other *Acinetobacter* species, such as *A. johnsonii*, *A. junii*, *A. lwoffii* **etc**- are rare and are mainly restricted to catheter related bloodstream infections (Detret-Tega *et al.*, 2007). These infections cause minimal mortality and their clinical course is usually benign, although life-threatening sepsis has been observed occasionally (Linde *et al.*, 2002). The most frequent clinical manifestations of nosocomial *A. baumannii* infection are ventilator-associated pneumonia and bloodstream infection, both of which are associated with considerable

morbidity and mortality, which can be as high as ~~30% to 52–60%~~ (Seifert *et al.*, 199~~56~~; Cisneros *et al.*, 2002; Wisplinghoff *et al.*, 2004).

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Recently, bacteraemia caused by *A. baumannii* is one of the infections with the highest mortality rate in hospitals (Joly-Guillou *et al.*, 2005). A survey by the Health Protection Agency in England found that patients with *Acinetobacter* bacteraemia were generally aged > 50 years,

~~that the majority of the patients were male, and that 5% of the patients~~
were hospitalized in general wards and 54% were in ICUs (Wisplinghoff *et al.*, 2000).

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Risk-factors have been defined in many studies, and are essentially the same as those identified for other opportunistic bacteria (Lee *et al.*, 2004; Falagas *et al.*, 2006; Baran *et al.*, 2008; Shih *et al.*, 2008; Baran *et al.*, 2008). Another study reported that sepsis and/or septic shock in 19% of patients with bacteraemia were caused by *A. baumannii* (Valero *et al.*, 2001). This observation also highlighted the true pathogenicity of *A. baumannii* strains, with a crude mortality rate of 42%.

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The infection rate of *A. baumannii* in Hospital USM (HUSM) intensive care unit ~~were was~~ shown to be higher than the one reported (19%) from Hospital UKM (another teaching hospital in Malaysia) by Rozaidi *et al.*, 2002. In our current study, we found that the prevalence of *A. baumannii* infection in Hospital Universiti Sains Malaysia (HUSM) is varyingies from year to year, and the overall prevalence of *Acinetobacter* infection in intensive care units was 12.65%. In 2005, 2006, 2007 and 2008 (up-to June)

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the prevalence rate was 18.92%, 29.89%, 26.42% and 26.01% respectively (Data collected from Infection Control Unit, HUSM). The prevalence of *A. baumannii* infection in intensive care units (General ICU, Neurosurgical ICU and Neonatal ICU) was higher compared to the general ward. The overall prevalence of *Acinetobacter* infection in intensive care units was 12.65%.

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1.2.1 Pathogenesis

A recent study (Smith *et al.*, 2007) has revealed that a large portion of the genome of *A. baumannii* consists of pathogenicity islands (PAIs). PAIs contain genes implicated in virulence, of which the largest appears to contain a type IV secretion apparatus. Type IV secretion systems have been shown to play an important role in other human pathogens, including *Bordetella pertussis*, *Legionella pneumophila*, *Brucella* spp. and *Helicobacter pylori* (Schmidt & Hensel, 2004). In the case of *A. baumannii*, this may be more important as PAI genes, like other virulence genes, respond to environmental stimuli and thus may only be expressed under stressful conditions.

Smith *et al.* (2007) also compared the genome sequence of *A. baumannii* with that of its closest sequenced relative, *the nonpathogenic non-pathogenic* *A. baylyi*, using the Artemis Comparison Tool (ACT) to identify *A. baumannii* virulence genes. They found that the most interesting differences between these two organisms lies in the 28 PAIs identified in *A. baumannii*. Many of the drug-resistance and potential virulence factors found in the *A. baumannii* genome reside on these islands, indicating that a large number of them are important factor for the pathogenesis of *A. baumannii*. This presumably comprises a small fraction of elements involved in *A. baumannii* pathogenesis, and thus,

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novel global approaches are essential to comprehensively understand the basic features of this organism in order to ultimately control the spread of *A. baumannii* infections and to develop effective counter-measures against this harmful pathogen.

Clinical isolate of *A. baumannii* is able to survive on abiotic surfaces (plastic or glass surfaces) and produce biofilm, a property that is most likely to be associated with the capacity of this pathogen to survive in hospital environments and medical devices, and cause severe infections in compromised patients (Tomaras *et al.*, 2003). Recently, there was another study performed in Korea that showed that *A. baumannii* has significant correlation with epithelial cell adherence because of the ability to form biofilm (Lee *et al.*, 2008). This is because cells growing in biofilms are highly resistant to the components of the human immune system and to numerous types of antimicrobial agents. The study also revealed that *A. baumannii* isolates carrying blaPER-1 showed a significantly higher capacity for epithelial cell adherence and biofilm formation when compared with *A. baumannii* isolates without blaPER-1 (Lee *et al.*, 2008, Loehfelm *et al.*, 2008).

Being a human pathogen, *A. baumannii* must be able to utilize host resources in order to survive. Iron is an important resource that is not readily available in the human host; rather, it is found complexed with iron binding molecules such as heme, lactoferrin, and transferrin. Bacteria survive and multiply under iron-limiting conditions, such as those found in natural and host environments, by expressing active systems that gather this essential micronutrient (Echenique *et al.*, 2001). A study was also performed

to check the iron uptake components of clinical isolates_s of *A. baumannii* showed that most of the clinical isolates_s contains *fatA*-like gene. This gene which is potentially involved in iron acquisition, can be located in different genomic regions in-for different *A. baumannii* isolates. and Disruption of the *fatA*-like gene indeed will impairs the iron acquisition phenotype of this strain, hence confirming its role in iron transport (Dorsey *et al.*, 2003).

The pathogenic determinants that have been reported so far for *A. baumannii* include a novel pilus assembly system involved in biofilm formation (Lee *et al.*, 2008; Tomaras *et al.*, 2003), an outer membrane protein (Omp38) that causes apoptosis in human epithelial cells (Choi *et al.*, 2005), and a polycistronic siderophore-mediated iron-acquisition system conserved between *A. baumannii* and *Vibrio anguillarum* (Dorsey *et al.*, 2003, Dorsey *et al.*, 2004).

1.2.41.2.2 Virulence factors

Although *Acinetobacter baumannii* are considered to be relatively low grade pathogens, certain characteristics of these organisms may enhance the virulence of strains involved in infections. These characteristics include: the presence of a polysaccharide capsule, formed by L-rahmnose, D-glucose, D-glucuronic acid and D-mannose, which probably render the surface of strains more hydrophilic, although hydrophobicity may be higher in isolated from catheters or tracheal devices (Joly Guillou *et al.*, 2005). The property of adhesion to human epithelial cells in the presence of fimbriae and/or capsular polysaccharides. The production of enzymes which may damage tissue lipids and the potentially toxic role of the lipopolysaccharide component of the cell wall and the presence of lipid A.

Nosocomial *A. baumannii* bacteraemia may cause severe clinical disease that is associated with a high mortality rate of up to 17% to 75% (Cisneros *et al.*, 1996; 2002). This opportunistic pathogen causes a wide variety of serious infections in humans, mostly in compromised patients. Recently, *A. baumannii* has emerged as an important pathogen among wounded soldiers, threatening civilian and military patients (Davis *et al.*, 2005; Scott *et al.*, 2007; Niu *et al.*, 2008). This opportunistic pathogen expresses a myriad of factors that could play a role in human pathogenesis. Among these factors are the attachment to and persistence on solid surfaces, the acquisition of essential nutrients such as iron, the adhesion to epithelial cells and their subsequent killing by apoptosis, and the production and/or secretion of enzymes and toxic products that damage host tissues. However, very little is known about the molecular nature of most of these processes and factors and almost nothing has been shown with regard to their role in bacterial virulence and the pathogenesis of serious infectious diseases. Fortunately, some of these gaps can now be filled by testing appropriate isogenic derivatives in relevant animal models that mimic the infections in humans, particularly the outcome of deadly pneumonia. Such an approach should provide new and relevant information on the virulence traits of this normally underestimated bacterial human pathogen.

A. baumannii infections probably involve numerous factors, including virulence determinants, which have yet to be investigated. *A. baumannii* began to spread rapidly among patients in intensive care units (ICUs) in the 1980s. But studies on *Acinetobacter* virulence factors are still at an elementary stage. Non-specific adherence factors, such as fimbriae, which help adherence to human gastric epithelial cells via adhesions, have

been described in *A. baumannii* (Lee *et al.*, 2006). It is known that, under iron-deficient conditions, bacterial growth can be accompanied by the production of receptors and iron-regulated catechol siderophores, which will, in turn, favour bacterial growth and the expression of virulence factors (Goel *et al.*, 2001).

Acinetobacter also can trigger gastritis including hypergastrinaemia and stimulation of cytokine release by the expression of virulence factors (Rathinavelu *et al.*, 2003).

Another neuropathological studies have demonstrated that amino acid sequence homology exists between a bovine prion sequence (RPVDQ) and an enzyme produced by *Acinetobacter* uridine diphosphate-N-acetylglucosamine-1-carboxyvinyl transferase, which also contains the RPVDQ sequence and could be potentially cross-reactive. As a consequence, an antibody response to the *Acinetobacter* sequence could influence the pathology of the disease (Wilson *et al.*, 2004).

Approximately 30% of *Acinetobacter* strains produce exopolysaccharide, which is a major virulence factor and is thought to protect bacteria from host defences resulting in cytotoxicity for phagocytic cells (Joly-Guillou *et al.*, 2005). In experimental studies, exopolysaccharide-producing strains of *Acinetobacter* have been shown to be more pathogenic than non-exopolysaccharide-producing strains, especially in polymicrobial infections with other species of higher virulence.

Quorum-sensing is another widespread regulatory mechanism among *A. baumannii* which is required for the later stages of biofilm maturation. At present, the

only known determinants required for biofilm formation in *A. baumannii* are the *csu*-encoded chaperone-usher pilus assembly system (Tomaras *et al.*, 2003) and the Bap protein (Loehfelm *et al.*, 2008). Recently another study showed that the *abaI*-directed quorum-sensing pathway is required for the later stages of biofilm maturation (Niu *et al.*, 2008). Quorum-sensing might be a central mechanism for auto-induction of multiple virulence factors in an opportunistic pathogen such as *A. baumannii*; ~~and this process should be studied for its clinical implications~~ (Joly-Guillou *et al.*, 2005).

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1.2.3 ~~5~~ Risk factors

Acinetobacter A. baumannii is an important cause of nosocomial infections in many hospitals, which is difficult to ~~both~~ control and treat because of its prolonged environmental survival and its ability to develop resistance to multiple antimicrobial agents (Bergogne-Berezin *et al.*, 1996; Cisneros *et al.*, 2005; ~~Yu-Chen Tseng~~ *et al.*, 2007; ~~Cisneros et al., 2005; Bergogne-Berezin et al., 1996~~). *A. baumannii* appears to have a propensity for developing antimicrobial resistance extremely rapidly. Moreover, this resistance is multiple, causing serious therapeutic problems (Cisneros *et al.*, 2002). Several studies were conducted to find the risk factors as bacteraemia caused by ~~multidrug-resistant A. baumannii (MDRAB)~~ leads to higher mortality and medical cost compared with non-MDRAB bacteraemia. Risk factors may vary between areas with endemic colonization and epidemic outbreaks of infection (Rello et al., 1999; Garcia-Garmendia et al., 2001; Mu-Jen Shih et al., 2008); ~~Garcia-Garmendia et al., 2001; Rello et al., 1999~~. From the previous studies of risk factors, it was found that longer duration of hospital stay until *A. baumannii* isolation, ICU admission, emergent surgical

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operation, total parenteral nutrition, invasive procedures such as central venous catheter, endotracheal tube, urinary catheter, or nasogastric tube, previous administration of carbapenems and previous exposure to broad-spectrum antibiotics have been identified as risk factors for acquisition of *A. baumannii*. ~~in numerous studies were significant risk factors for *A. baumannii* infections~~ ([Garcia-Garmendia et al., 2001](#); [Joly-Guillou et al., 2005](#); [Gulseren-Baran et al., 2008](#); [Joly Guillou et al., 2005](#); [Garcia Garmendia et al., 2001](#)).

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The risk factors that predispose individuals to the acquisition of ~~and infection with~~ *A. baumannii* are similar to those that have been identified for other [MDR](#)-[multi-drug resistant](#) organisms. Risk factors that are specific for a particular setting have also been identified, such as the hydrotherapy that is used to treat burn patients and the pulsatile lavage treatment that is used for wound treatment ([Maragakis et al., 2004](#); [Wisplinghoff et al., 1999](#); [Maragakis et al., 2004](#)). The most frequent clinical manifestations of nosocomial *A. baumannii* infection are ventilator-associated pneumonia and bloodstream infection, both of which are associated with considerable morbidity and mortality, which can be as high as 52% ([Seifert et al., 1995](#); [Cisneros et al., 2002](#); [Seifert et al., 1995](#)).

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1.2.46 Prevalence

The prevalence of nosocomial bloodstream infections due to *Acinetobacter* ~~A.~~ *baumannii* currently has become a public health problem in many countries ~~ranges~~ ranging from 2% to 10% of all gram-negative bacterial infections in Europe (Hanberger et al., 1999) and account for about 2.5% of them in the United States (Jones et al.,

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2004).

The incidence of severe infection caused by *Acinetobacter* species has been increasing. For example, National Nosocomial Infection Survey data for US intensive care units indicate that *Acinetobacter* species caused 6.9% of hospital-acquired pneumonia in 2003, compared with 1.4% in 1975. The rates of bloodstream infection, surgical site infection, and urinary tract infection have also increased during this period (from 1.8% to 2.4%, 0.5% to 2.1%, and 0.6% to 1.6%, respectively (Gaynes *et al.*, 2005).

A. baumannii exhibits a remarkable ability to rapidly develop antibiotic resistance that led to multidrug resistance (MDR) within a few decades (Bergogne-Berezin *et al.*, 1996). To date, some strains of *A. baumannii* have become resistant to almost all currently available antibacterial agents (Van Leeuwen *et al.*, 2004), mostly through the acquisition of plasmids (Joshi *et al.*, 2003), transposons (Smith *et al.*, 2007), or integrons carrying clusters of genes encoding resistance to several antibiotic families (Segal *et al.*, 2003; Poirel *et al.*, 2003) at once.

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1.2.71.2.5 Antimicrobial susceptibility and resistant mechanisms

Acinetobacter—*baumannii* is attracting much attention owing to the increase in antimicrobial resistance and occurrence of strains that are resistant to virtually all available drugs (Perez *et al.*, 2007). This organism is generally intrinsically resistant to a number of commonly used antibiotics, including aminopenicillins, first and second generation cephalosporins and chloramphenicol (Vila *et al.*, 1993; Seifert *et al.*, 1995).

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It also has a remarkable capacity to acquire mechanisms that confer resistance to broad-spectrum-lactams, aminoglycosides, fluoroquinolones and tetracyclines⁴. *A. baumannii* exhibits a remarkable ability to rapidly develop antibiotic resistance that led to multidrug resistance within a few decades (Bergogne Berezin *et al.*, 1996). To date, some strains of *A. baumannii* have become resistant to almost all currently available antibacterial agents (Looveren *et al.*, 2004), mostly through the acquisition of plasmids (Joshi *et al.*, 2003), transposons (Smith *et al.*, 2007), or integrons carrying clusters of genes encoding resistance to several antibiotic families (Segal *et al.*, 2003; Poirel *et al.*, 2003).

Numerous studies have suggested an upward trend in strains of *A. baumannii* that are resistant to these agents. However, because of the scarcity of large-scale surveillance studies from the 1970s to the 1990s and the difficulties in comparing local reports, such trends are difficult to quantify on a global level. Resistance rates can vary according to the country and the individual hospital, and depend on biological, epidemiological or methodical factors (Seifert-Wisplinghoff *et al.*, 2007⁶). Recently, resistance to polymyxins and tigecycline has been described, which indicates that *A. baumannii* can cause infections that are fully refractory to the currently available antimicrobial drugs (Li *et al.*, 2006; Pleg *et al.*, 2007).

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The resistance of *A. baumannii* to antimicrobial agents is mediated by all of the major resistance mechanisms that are known to occur in bacteria, including modification of target sites, enzymatic inactivation, active efflux and decreased influx of drugs (Poirel *et al.*, 2003). Beta-β-lactamases are the most diverse group of enzymes that are associated

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with resistance, and more than 50 different enzymes, or their allelic forms, have been identified so far in *A. baumannii* (Dijkshoorn *et al.*, 2007). In a previous study-, *aac(6')*-*Ib* and *aac(6')*-*Ih* have been identified as the most prevalent plasmid-mediated *aac(6')*-*I* genes_among *A. baumannii* strains through which aminoglycoside resistance can be attributed to at least nine distinct modifying enzymes with different combinations in some strains (Doi *et al.*, 2004). In_Another study reported that resistance to tetracyclines has been associated with *tet* (A) and *tet* (B) genes that encode tetracycline-specific efflux pumps (Huys *et al.*, 2005). ISAbal ~~is-was~~ also thought to have a key role in some carbapenem-resistant strains by enhancing the expression of the intrinsic OXA-51-like carbapenemases (Turton *et al.*, 2006). Another chromosomal system that is typically found in *A. baumannii* is the AdeABC efflux system (Magnet *et al.*, 2001). Reduced susceptibility to carbapenems has also been associated with the modification of penicillin-binding proteins and porins or with ~~upregulation~~up regulation of the AdeABC efflux system, which might result in high-level carbapenem resistance in *A. baumannii* (Bou *et al.*, 2004). There was_Another study conducted by Felipe Fernandez Cuenca and colleagues in 2003 showed that production of β-lactamases ~~of pl 6.3 and 7.0~~ and reduced expression of PBP 2 (penicillin-binding protein ~~b~~Biotype 2) are the most frequently observed mechanisms of resistance to carbapenems (~~Fernandez-Cuenca et al., 2003~~)

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1.2.8 1.2.6 Treatment

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Recently, according to the infectious disease society of America (IDSA), *A. baumannii* is considered as one of the three increasingly problematic Gram-negative pathogens (Talbot-GH, 2008). MDRAB infections are difficult and costly to treat. A study at a public teaching hospital found that the mean total hospital cost of patients who acquired MDRAB was \$USD 98,575 higher than that of control patients who had identical burn severity of illness indices (Wilson *et al.*, 2004).

The incidence of severe infection caused by *Acinetobacter* species has been increasing. For example, National Nosocomial Infection Survey data for US intensive care units indicate that *Acinetobacter* species caused 6.9% of cases of hospital-acquired pneumonia in 2003, compared with 1.4% in 1975. The rates of bloodstream infection, surgical-site infection, and urinary tract infection have also increased during this period (from 1.8% to 2.4%, 0.5% to 2.1%, and 0.6% to 1.6%, respectively (Gaynes *et al.*, 2005).

Therapy-Treatment of *Acinetobacter* infection has been complicated by increasing resistance due to aminoglycoside-modifying enzymes, ESBLs, carbapenemases, or changes in outer-membrane proteins and penicillin-binding proteins (Gales-Levin *et al.*, 2002). In some parts of the United States, many isolates are now resistant to all aminoglycosides, cephalosporins, and fluoroquinolones (Landman *et al.*, 2002). The carbapenems and combinations of a β-lactam with a β-lactamase inhibitor, such as ampicillin-sulbactam, retain useful activity, but resistance rates are increasing (Quale *et al.*, 2003). In a recent study (Talbot, 2008), data showed that for the carbapenems, which have been demonstrated to have the greatest inherent activity against *A. baumannii*, the frequency of resistance has increased by 30%, from 9% to 39% (p <

0.01). The rate of resistance to fluoroquinolones increased from 50% to 73%, and to β-lactams from 39% to 66% ($p < 0.01$ for each comparison). These changes in the epidemiology and resistance rates of *A. baumannii* have led clinicians to adopt therapeutic options, such as colistimethate sodium ('colistin'—also known as polymixin E), the use of which had previously been abandoned in clinical use because of an unacceptably high rate of renal toxicity (Falagas *et al.*, 2005; Li *et al.*, 2006).

Currently, several studies have tested the *in vitro* activity of tigecycline, a semi-synthetic tetracycline (glycylcycline) against *A. baumannii* and reported good bacteriostatic activity (Seifert-Curcio *et al.*, 2008). However, current evidence casts doubt on the role of tigecycline as a treatment for MDR *A. baumannii* infection, with reports showing off-high tigecycline resistance (Navon-Venezia *et al.*, 2007; Peleg *et al.*, 2007; Reid *et al.*, 2007; Ruzin *et al.*, 2007; Peleg *et al.*, 2007; Navon-Venezia *et al.*, 2007). The ability of *Acinetobacter* to rapidly acquired resistance to this new glycylcycline antimicrobial is cause for concern and adds further stimulus for the discovery of newer antimicrobials with activities against this problematic organism (Peleg *et al.*, 2007). Recently, there was a study performed in USA to check the activities of tigecycline in combination with other antimicrobials. But-However, the safety and tolerability associated with the elevated dosages for tigecycline are-were not known-determined (Scheetz *et al.*, 2007). Because-So far no clinical trial has been performed and the exact role of tigecycline in therapy of *A. baumannii* infection remains to be defined (Talbot, 2008).

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Bloodstream infections with *A. baumannii* are occurring with increasing frequency, resulting in significant morbidity and mortality ranging from 8% to 43% (Scheetz *et al.*, 2007). Therefore, clinicians must resort to empirical combination therapy, which has an unproven utility and therapeutic failures and relapses are anticipated. Recently, BAL 30376 (Basilea Pharmaceutica Ltd, Switzerland), a novel β -lactum/ β -lactamase inhibitor combination, represents an interesting potential approach to therapy of multidrug-resistant *A. baumannii* (Talbot, 2008). But However, clinical study is not yet been conducted on this therapy. In conclusion, we can say as noted by the IDSA, “*A. baumannii* is a prime example of mismatch between unmet medical needs and the current antimicrobial research and development pipeline”.

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1.3.1 Rationale of the study

Acinetobacter *A. baumannii* appears to have a propensity for developing antimicrobial resistance extremely rapidly (Cisneros *et al.*, 2002). Moreover, resistance involves multiple drugs and causes serious therapeutic problems. The reason that antibiotic resistance leads to adverse outcomes is presumably because of an increased likelihood that antibiotic therapy will be ineffective or suboptimal (Lee *et al.*, 2007). A higher sepsis-related mortality rate among patients with multidrug-resistant *A. baumannii* bacteraemia, compared with that for patients with non-multidrug-resistant *A. baumannii* bacteraemia is likely associated with a longer delay in the initiation of appropriate therapy. Several studies have described the relationship between receipt of appropriate therapy and a favorable outcome for the patients with nosocomial bloodstream infections (Ibrahim *et al.*, 2000; Zaragoza *et al.*, 2003). Nosocomial bacteraemia due to *MDRAB multidrug-resistant A. baumannii* is associated

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with increased in medical costs, prolonged hospitalization, and an increased in-mortality rate.

Major obstacles in controlling this pathogen are the high contagiousness of the disease⁺ and the emergence of multi-resistance characteristics to the commonly prescribed antibiotics. As developing antimicrobial resistance to multiple antibiotics causes severe serious therapeutic problems⁺, it is crucial to develop a rapid method for identifying the bacteria in order to limit and control outbreaks. Immediate identification of the pathogen in clinical samples is critical to ensure proper clinical treatment, management of the patient and for epidemiological investigations. Current laboratory diagnostic method used to diagnose *Acinetobacter* infection relied on the time-consuming growth in culture media, followed by isolation, biochemical and serological identification. The relatively low sensitivity and the difficulty in performing the current diagnostic procedure have called for an alternative diagnostic method for the early identification of the bacteria. A rapid, simple and reliable diagnostic test is highly desired. One such method involves detection of specific antibodies in clinical specimens. There is a need for the development of next generation immunoassay technologies which provide a more rapid, sensitive and portable assays. The immunochemical technology, which is based on the membrane-based antibody assays, has been shown to be a potential tool for the diagnosis of pathogens (Smits *et al.*, 2003; Lammie *et al.*, 2004). The advantages of the immunochemical test over culture method are rapidity, simplicity, do not require expensive equipment, do not require cold chain for transportation, enhanced sensitivity and specificity for early diagnosis and the test can be performed at the point

of care. However, there is no report on diagnostic applications employing the immunochromatography concept that have been developed for diagnosis of *A. baumannii* infection. A rapid and reliable diagnostic assay would significantly improve effective management of the disease especially among young children and particularly in patients who are critically ill or immunocompromised. To control outbreaks of the infection caused by *A. baumannii* and to prevent further complications, it is often necessary to treat patients with specific antibodies-antibiotics at the early stage. This is also very important to reduce the morbidity and mortality and the selection of appropriate antibiotics to control the nosocomial infection caused by *A. baumannii*. Current global diagnostic trend is moving towards rapid immunochemistry-ICT platform, there is a need to strategically convert to this test to achieve a more rapid laboratory diagnosis. Thus, efforts to be taken to minimize the delay in the administration of appropriate antibiotic therapy are essential, as are techniques to facilitate the earlier identification of drug-resistant organisms like *A. baumannii*.

1.4 Objective of the study

Nosocomial bacteraemia due to multidrug resistant *A. baumannii*MDRAB is associated with increased medical costs, prolonged hospitalization, and an increased in mortality rate. Thus, efforts to minimize the delay in the administration of appropriate antibiotic therapy are essential, as are techniques to facilitate the earlier identification of drug-resistant organism like *A. baumannii*. The ability to produce indigenous test for *A. baumannii* that are novel, specific yet cost effective would now provide a huge impact on public health management not only in Malaysia but all over the world. Thus, the main aim of this study is to find outdemonstrate the presence of a specific protein

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