DEMONSTRATION OF ANTIGENIC AND SPECIFIC SURFACE ASSOCIATED HEAT SHOCK PROTEIN(S) OF *Acinetobacter baumannii*

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by

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LIST OF ABBREVIATIONS

AP	Alkaline Phosphatase
ATCC	American Type Culture Collection
ESBL	Extended spectrum beta-lactamases
HSP	Heat Shock Protein
ICT	Immunochromatography
kDa	Kilo Dalton
MALDI-ToF	Matrix-assisted Laser Desorption/Ionization-Time of Flight
MDR	Multidrug-resistance
MW	Molecular Weight
MDRAB	Multidrug-resistance *Acinetobacter baumannii*
MRSA	Methicillin-resistant Staphylococcus aureus
NaOH	Sodium Hydroxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPSP	Pusat Pengajian Sains Perubatan
PVDF	Polyvinylidene Difluoride
SAP	Surface-associate Protein
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
SIM	Sulfide indole motility media
TEMED	N,N,N’,N’-tetramethylethylenediamine
TSB	Tryptic Soy Broth
TSI	Triple sugar ion agar

VAP	Ventilator-associated pneumonia
°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microliter
mg	Milligram
ml	Millilitre
g	Gram
h	Hour
V	Volt

DEMONSTRASI KEHADIRAN PROTEIN BERKAIT PERMUKAAN KEJUTAN HABA YANG ANTIGENIK DAN SPESIFIK BAGI *Acinetobacter baumannii*

ABSTRAK

Acinetobacter baumannii adalah patogen nosokomial yang penting dan menyebabkan kadar mortaliti yang tinggi. Hal ini merupakan kebimbangan utama dalam kes jangkitan yang diperoleh di hospital di seluruh dunia. Sehingga kini, *Acinetobacter baumannii* telah mempamerkan peningkatan yang berterusan dalam kadar rintangan terhadap semua antibiotik barisan pertama. Keadaan ini menyebabkan pilihan agen antimikrob untuk merawat jangkitan *Acinetobacter baumannii* adalah terhad. Pada masa ini, kaedah piawai yang digunakan untuk mengenal pasti spesies *Acinetobacter* adalah hibridasi DNA-DNA, manakala dua kaedah yang disyorkan dan telah diterima secara meluas untuk pengenalpastian spesies *Acinetobacter* adalah kaedah (amplified rDNA restriction analysis) ARDRA dan (amplified fragment length polymorphism) ALFP. Walau bagaimanapun, semua kaedah tersebut terlalu rumit untuk digunakan sebagai ujian rutin di makmal diagnostik. Oleh yang demikian, pembangunan ujian pengesanan awal bagi *Acinetobacter baumannii* amat diperlukan untuk mengurangkan masa pengesanan jangkitan *Acinetobacter baumannii* pada pesakit. Pembangunan ujian diagnostik yang spesifik dan sensitif memerlukan penemuan biomarker yang tidak bertindakbalas silang dengan bakteria lain dan spesifik hanya untuk *Acinetobacter baumannii*. Protein kejutan haba (HSP) adalah protein yang dihasilkan oleh bakteria semasa berada di dalam keadaan persekitaran yang tertekan dan protein ini mempunyai potensi sebagai biomarker untuk digunakan dalam diagnostik. Justeru itu, tujuan kajian

ini adalah untuk mengesan kehadiran HSP dan biomarker daripada protein berkaitan permukaan (SAP) daripada *Acinetobacter baumannii*. Dalam kajian ini, profil SAP daripada strain ATCC 19606 dan strain klinikal *Acinetobacter baumannii* telah dipencil menggunakan teknik SDS-PAGE. Kajian ini menunjukkan tahap ekspresi protein SAP *Acinetobacter baumannii* berubah dengan peningkatan suhu. Profil SAP yang diekspreskan pada suhu 37°C telah dibandingkan dengan profil pada suhu 38.5°C dan 41°C untuk mengenal pasti kesan perubahan suhu terhadap ekspresi SAP. Protein tersebut seterusnya dianalisis dengan kaedah Western blot menggunakan serum daripada pesakit yang dijangkiti dengan *Acinetobacter baumannii* serta jangkitan bakteria lain. Keputusan kajian menunjukkan pelbagai jalur antigen telah dikesan apabila diuji dengan sera daripada pesakit yang dijangkiti dengan *Acinetobacter baumannii* (diinkubasi pada 37°C dan 41°C) terhadap antibodi kelas IgA, IgM dan IgG. Tindakbalas silang protein antigenik diperiksa menggunakan sera pesakit yang dijangkiti *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp, *Staphylococcus aureus*, *Enterococcus faecalis* dan Methicillin-resistant Staphylococcus aureus (MRSA). Lima protein antigenik telah dipilih untuk pengenalpastian dan pencirian selanjutnya menggunakan kaedah MALDI-ToF. Protein 96.7kDa, 62.0kDa, 37.9kDa, 25.0kDa dan 16.0kDa telah dikenalpasti sebagai catalase HPII, Hsp60 chaperonin, phosphate ABC transporter substrate binding protein, Y*cei*-like protein, and Lipopolysaccharide export system protein LptA. Peningkatan dalam tahap ungkapan protein-protein yang antigenic tersebut berkemungkinan menyumbang kepada mekanisme ketahanan bakteria tersebut pada suhu yang tinggi dalam tubuh pesakit, dan mempunyai potensi sebagai biomarker untuk pembangunan ujian diagnostik dan juga bagi pembangunan vaksin terhadap bakteria tersebut.

DEMONSTRATION OF ANTIGENIC AND SPECIFIC SURFACE ASSOCIATED HEAT SHOCK PROTEIN(S) OF *Acinetobacter baumannii*

ABSTRACT

Acinetobacter baumannii is an important nosocomial pathogen associated with high mortality. It is a major concern of hospital acquired infection (HAI) worldwide. Until recently, the resistance of *Acinetobacter baumannii* has increased steadily against all first-line antibiotics. Due to this situation, the choice of antimicrobial agents to treat *Acinetobacter baumannii* infections is limited. Currently, the gold standard method for identification of *Acinetobacter* species is the DNA-DNA hybridization while the two recommended methods that are widely accepted for identification of *Acinetobacter* species are amplified rDNA restriction analysis (ARDRA) and amplified fragment length polymorphism (ALFP). However, these methods are too laborious for everyday diagnostic use. Thus, there is a need to develop an early detection of *Acinetobacter baumannii* to reduce the time consuming in detecting the infection in patients. Development of a specific and sensitive diagnostic test requires discovery of biomarker(s) which does not cross react with other bacteria and specific only to *Acinetobacter baumannii*. Heat shock proteins (HSP) are proteins that expressed in bacteria during stress environment and these proteins have potential as biomarker in diagnostic field. Thus, the aim of this study is to detect the presence of HSPs and biomarker(s) in the surface associated proteins (SAPs) of *Acinetobacter baumannii*. The SAPs profile from the ATCC 19606 strain and clinical isolate of *Acinetobacter baumannii* were demonstrated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This study demonstrated that the expression level of

SAPs of *Acinetobacter baumannii* varies with increasing temperature. The SAPs profiles expressed at 37°C was compared with 38.5°C and 41°C to assess the effect of temperatures on the expression of the SAPs. The protein was subjected to Western blot using serum from patients infected with *Acinetobacter baumannii* as well other bacteremia infections. Result of this study demonstrated various antigenic bands detected when probed with sera from patients with *Acinetobacter baumannii* infections (incubated at 37°C and 41°C) against IgA, IgM and IgG isotypes. All the antigenic bands were checked for cross reaction using sera from patients infected with *Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella* spp, *Staphylococcus aureus, Enterococcus faecalis and* methicillin-resistant *Staphylococcus aureus* (MRSA). Five specific and antigenic proteins were selected for further identification by MALDI-ToF analysis. The protein 96.7kDa, 62.0kDa, 37.9kDa, 25.0kDa and 16.0kDa from *Acinetobacter baumannii* were identified as catalase HPII, Hsp60 chaperonin, phosphate ABC transporter substrate binding protein, Y*cei*-like protein, and Lipopolysaccharide export system protein LptA, respectively. The increased expression of antigenicity level of these proteins probably is a survival mechanism of the bacteria at higher temperature in the host body and could be potential diagnostic biomarkers for diagnostic and vaccine development.

CHAPTER 1

INTRODUCTION

Over the last few decades, *Acinetobacter* has undergone significant taxonomic modification. The most important representative, *Acinetobacter baumannii*, has emerged as one of the most troublesome pathogens for nosocomial and community-acquired infection (Gootz & Marra, 2008). According to Centres for Disease Control and Prevention (CDC), USA, *A. baumannii* is a major cause of hospital outbreaks, particularly in intensive care units, accounting for approximately 80% of the reported hospital infections (Bigot & Salcedo, 2017). Its increasing capacity to resist a broad range of antimicrobial agents has placed *A. baumannii* as a serious global health threat. In a hospital setting, this opportunistic pathogen is usually associated with bacteremia, pneumonia/ventilator-associated pneumonia (VAP), and meningitis or urinary tract infections. Patients that have undergone major surgery or presented with severe burns are at particular risk of this infection (Bigot & Salcedo, 2017).

1.1 MICROBIOLOGY

1.1.1 Historical perspective of the genus *Acinetobacter*

In early 20th century, in 1911, a Dutch microbiologist's Beijerinck has described an organism as *Micrococcus calcoaceticus* in which it has been isolated from soil and was enriched in a medium containing calcium-acetate mineral medium (La Riviere, 1997). After the following decade, the similar organism was described and assigned to at least 15 genera and species including *Diplococcus mucosus*, *Micrococcus calcoaceticus*, *Alcaligenes haemolysans*, *Mima poltmorpha*, *Moraxella Iwoffi*, *Harellea vaginicola*, *Bacterium anitratum*, *Morexella Iwoffi var. glucidolytica*, *Neisseria winogradskyi*, *Achromobacter anitratus* and *Achromobacter mucosus*.

It is until 1968 the genus designation become widely accepted the comprehensive survey suggested by Baumann and colleagues which concluded the different species listed above belong to a single genus. They proposed *Acinetobacter* as the genus name (Peleg *et al*., 2008).

1.1.2 Taxonomy

Currently, *Acinetobacter* genus is characterized as strictly aerobic, non-motile, Gram-negative, non-fermentative, oxidase-negative, catalase-positive organism (Gootz & Marra, 2008) and it is non-fastidious bacteria with a DNA G+C content of 39% to 47% (Peleg *et al.,* 2008). To the recent taxonomic data, it was put forward that the genus *Acinetobacter* should be categorized in the new family of *Moraxellaceae* within the order *Gammaproteobacteria,* which includes genera of *Moraxella, Acinetobacter, Psychrobacter* and related organism (Peleg e*t al.,* 2008). After the long and complicated history of genus, the designation of *Acinetobacter* taxonomy as being distinct from *Moraxellaceae* was achieved in 1986 by Bouvet and *Grimont* (Peleg *et al.*, 2008). There are currently 32 *Acinetobacter* named and un-named species. Four of the species, *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, are referred as *Acinetobacter calcoaceticus-A. baumannii* complex (Gerner-Smidt *et al.,* 1991; Gerner-Smidt, 1992). This is due to their interrelatedness and difficulties to differentiate them from each other by phenotypic properties. However, these group species are not primarily involved in vast majority clinical pathogenesis, i.e., *A. baumannii,* and *Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU, but also in environmental species *A. calcoaceticus*, that is usually recovered from soil and water which has not been implicated in serious clinical disease. Therefore, since *A. calcoaceticus* is an

environmental species, the given designation named *Acinetobacter calcoaceticus- A. baumannii* complex is not suitable in clinical context (Peleg *et al.,* 2008).

1.1.3 Natural habitats

Genus of *Acinetobacter* was known as ubiquitous organism since they can be recovered by enrichment culture from almost environmental sample like water surface and soil. But, not all *Acinetobacter* have their natural habitats in the environment. *Acinetobacter* are part of the human skin flora and most of *Acinetobacter* strains that were recovered from human clinical specimen has at least some significant as human pathogen (Seifert *et al.,* 1994). Because of the widespread presence of *Acinetobacter* in different ecological niches, there were equally misleading concept that *A. baumannii* is a normal component of the human flora. Hence, based on the basis of the ecology, epidemiology, and antibiotic phenotype of different isolates, (Towner, 2009) have proposed that *A. baumannii* and closely related members of the *A. baumannii* complex, was represented by strains isolated from medical environments and equipment, medical personnel, and hospitalized patients. In general, these isolates tend to be resistant to multiple antibiotics (McConnell *et al.,* 2013).

In other study, it was reported that species *A. baumannii* was found rarely on human skin 0.5% and 3% (Seifert *et al.,* 1997; Berlau *et al.,* 1999) and in human faeces 0.8% (Dijkshoorn *et al.,* 2005). In Europe, the carrier rate of *A. baumannii* in the community is rather low. Although *A. baumannii* has been found in soil samples in Hong Kong and on vegetables in the United Kingdom, *A. baumannii* does not appear to be a typical environmental organism because there is not enough data to determine if the occurrence of severe community-acquired *A. baumannii* infections that have been observed in

tropical climates may be associated with an environmental source (Leung *et al.,* 2006; Wang *et al.,* 2002). A study reported that *A. baumannii* was occasionally found in infected animal (Vaneechoutte *et al.,* 2000) and then there is speculation of the finding of recovered 22% of *A. baumannii* from body lice sample from homeless people might be the result of clinically silent bacteremia in these people (La Scola & Raoult, 2004).

1.1.4 Growth and cultural characteristics

Acinetobacter species of human origin grow well on solid media that are routinely used by clinical microbiology laboratories such as MacConkey agar and sheep blood agar incubated at 37°C temperature. Characteristic of the colonies on 5% sheep blood agar are smooth, opaque colonies, in which some isolate are β-haemolytic. Colonies on MacConkey agar are pinkish and the agar did not change colour which indicate the bacteria is non-lactose fermenting.

1.2 Species identification

1.2.1 Physiology and morphology

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

1.2.2 Biochemical characteristic

A. baumannii is identified as non-motile, catalase-positive, oxidase-negative and non-fermenting. On Triple Sugar Iron (TSI) agar, it shows alkaline slant and neutral butt and it does not produce gas (H_2S). The phenotypic characteristics of *Acinetobacter* identified by biochemical test were shown as in Figure 1.1.

Name of test	*Acinetobacter* species				
	Acb. complex	*A. lwoffii*	*A. haemolyticus*	*A. junii*	*A. radioresistens*
Gram staining	Gram negative cocci or coccobacilli				
Catalase	+	+	+	+	+
Oxidase	–	–	–	–	–
Motility	–	–	–	–	–
Urease	V	V	–	–	–
Citrate	+	–	+	+	–
OF glucose	+	–	V	–	–
Nitrate reduction test	–	–	–	–	–
Hemolysis	–	–	+	–	–
Gelatin hydrolysis	–	–	+	–	–
Growth at 42°C	+	–	–	–	–
Chloramphenicol sensitivity	R	S	R	R	R
Arginine hydrolysis	+	–	+	+	+

V: Variable, S: Sensitive, R: Resistant, *A. lwoffii: Acinetobacter lwoffii, Acb: Acinetobacter calcoaceticus-baumannii, A. haemolyticus: Acinetobacter hemolyticus, A. junii: Acinetobacter junii, A. radioresistens: Acinetobacter radioresistens*, OF: Oxidation-fermentation

Figure1.1 Phenotypic characteristic of *Acinetobacter* (Adopted from Gupta *et al.*, 2015)

1.2.3 Molecular identification

Various genotypic methods has been developed and validated for *A. baumannii* identification to distinguish individual genomic species including tRNA spacer fingerprinting (Ehrenstein *et al.*, 1996), AFLP (Janssen *et al*,. 1997), ARDRA (Dijkshoorn *et al.*, 1998), Matrix-assisted Laser Desorption Time-of-Flight (MALDI-ToF) technique (Marí-Almirall *et al.*, 2017) and DNA-DNA hybridization (Visca *et al*., 2011). Specific gene sequences can also be used, including the intergenic spacer (ITS) region between the 16S–23S rRNA genes (Chang *et al.*, 2005), recA (Krawczyk *et al.*, 2002), rpoB (Gundi *et al.*, 2009) and gyrB (Higgins *et al.*, 2010), although not all of these methods successfully discriminate among members of the *A. baumannii* group. In addition, most of these methodologies are laborious, time consuming, require special

skills and are unsuitable for use in routine clinical identification. Among all those identification methods developed, DNA-DNA hybridization are the gold standard method for identification of *Acinetobacter* species while ARDRA and ALFP are the two recommended methods that are widely accepted for identification of *Acinetobacter* species (Yamamoto *et al.,* 1999).

According to the current taxonomy, the genus *Acinetobacter* includes 27 valid species and the most recent addition were *Acinetobacter indicus* (Malhotra *et al.,* 2012) and nine genomic species of *Acinetobacter* genus identified by genomic methods DNA-DNA hybridization, all of which encompass strains found in a wide range of ecological niches (McConnell *et al.,* 2013). However, *A. baumannii,* genomic species 3 and 13TU, which were recently renamed *Acinetobacter pittii* and *Acinetobacter nosocomialis* respectively were the most medically relevant species belong to the *A. baumannii* complex (Nemec *et al.,* 2011) as they are highly similar from a phenotypic point of view as well as by DNA-DNA hybridization (Dijkshoorn *et al.,* 2007; Peleg *et al.,* 2008). In fact, *A. pittii* and *A. nosocomialis* are often mistakenly identified as *A. baumannii* by routine commercial systems as they cannot be differentiated by currently available identification systems such as API and VITEK because they are so much alike (Ferreira *et al.,* 2010).

1.3 Clinical significant of *Acinetobacter baumannii*

Acinetobacter baumannii is an important nosocomial pathogen that has been implicated in various infections occurs most typically in critically ill patients receiving mechanical ventilation in the intensive care units. Studies and a systematic reviews have reported that nosocomial infections with *A. baumannii* is associated with increased in mortality

rates (Falagas *et al*., 2006; Abbo *et al*., 2007; Falagas & Rafailidis, 2007; Lee *et al*., 2007), while community-acquired pneumonia caused by *A. baumannii* much less frequent than nosocomial infection (Anstey *et al*., 1992, 2002; Chen *et al*., 2001; Leung *et al*., 2006).

Hospital-acquired infections (HAIs) caused by *A. baumannii* include bloodstream infections, VAP, skin and soft-tissue infections, wound infections, respiratory and urinary tract infections, endocarditis, secondary meningitis and others related diseases (Lee *et al.,* 2008). The most frequent clinical manifestations of nosocomial *A. baumannii* infection were VAP and bloodstream infections (Seifert *et al.,* 1995; Cisneros *et al.,* 2002; Wisplinghoff *et al.,* 2004). It was said that VAP with mortality rate reported to be between 40% and 70% (Fagon *et al*., 1996; Garnacho *et al*., 2003), were caused by colonization of *A. baumannii* from the airway via environmental exposure, and it was followed by the development of pneumonia in the patients (Dijkshoorn *et al.,* 2007). While the most common sources of *A. baumannii* bloodstream infections were lower respiratory tract infections and intravascular devices (Seifert *et al.,* 1995; Cisneros *et al.,* 1996; Jang *et al.,* 2009; Jung *et al.,* 2010) and it was reported that the mortality rates for *A. baumannii* bloodstream infections to be between 28% and 43% (Seifert *et al*., 1995; Wisplinghoff *et al*., 2004). Risk factors associated with acquiring *A. baumannii* bloodstream infections include immunosuppression, ventilator use associated with respiratory failure, previous antibiotic therapy, colonization with *A. baumannii*, and invasive procedures (Jang *et al*., 2009; Jung *et al*., 2010).

A. baumannii has also emerged as an important cause of burn infection in military personnel as evidenced by a recent report characterizing bacterial infections in a military burn unit which identified *A. baumannii* as the most common cause of burn site

infection with 53% of isolates demonstrating multidrug resistance (Keen *et al.*, 2010). Burn infection can be especially problematic as it can delay wound healing and lead to failure of skin grafts, and wound site colonization can progress to infection of the underlying tissue and subsequent systemic spread of the bacteria (Trottier *et al.*, 2007). *A. baumannii* is an increasingly important cause of meningitis, with the majority of cases occurring in patients recovering from neurosurgical procedures (Cascio *et al*., 2010) although there were rare cases of *A. baumannii* meningitis have been reported (Ozaki *et al*., 2009). Clinical features of *A. baumannii* meningitis are consistent with those of bacterial meningitis caused by other organisms and include fever, altered consciousness, headache, and seizure (Rodríguez Guardado *et al*., 2008). Mortality rates associated with *A. baumannii* meningitis are difficult to estimate because of a limited number of studies with adequately sized study populations. As with other types of infections caused by this pathogen, the emergence of drug resistance has posed an increasing challenge to clinicians (Wareham *et al*., 2008).

1.3.1 Pathogenesis and immune responses

Acinetobacter baumannii were able to withstand desiccation and disinfection to persist in the hospital environment. This ability contributes to the strength of *A. baumannii* tendency to form biofilms on a number of abiotic surfaces, including catheters, ventilators, and other medical devices, enhancing bacterial transmission (Pour *et al*., 2011). The immune responses were mainly dependent on how the host interacts with pathogen. *A. baumannii* is a versatile pathogen that can adhere to and invade numerous cell types, yet different cell types display varying degrees of susceptibility to invasion (Choi *et al.*, 2008).

It was reported that the invasion of *A. baumannii* into vertebrates host does not go undetected due to the vigilant monitoring by rapid immune response as the recognition by sentinel receptors triggers the immune response. The innate immune receptor on the host cells will detect the bacterial pathogen-associated molecular patterns (PAMPs) that will give intracellular signalling pathways that promote transcription, processing and secretion of inflammatory mediators which will response to stimulates chemokine secretion and subsequent recruitment of immune cells to the site of infection in both pneumonia and septicaemia infection. Although there are multiple inflammatory signalling cascades leading to cytokine and chemokine secretion, *A. baumannii* PAMPs are known to activate the NF-κB and MAPK pathways (March *et al.,* 2010). This process will induces the release of numerous chemokines, including macrophage inflammatory protein 2 (MIP-2), monocyte chemoattractant protein (MCP-1), and keratinocyte-derived chemokine (KC)/IL-8, and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Knapp *et al.,* 2006; van Faassen *et al.,* 2007). Natural killer cells participate in neutrophil recruitment through increased expression of KC/IL-8 (Tsuchiya *et al.,* 2012). Lymphocytes and granulocytes produce a number of antimicrobial factors in response to infection and killing of *A. baumannii*, including defensins, cathelicidins, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Qiu *et al.,* 2009; March *et al.,* 2010).

Garcia *et al* has reported that the lipopolysaccharide (LPS) of *A. baumannii* is highly immunostimulatory (Garcia *et al.,* 1999). Of the three components of LPS (lipid A, core polysaccharide, and O-antigen), lipid A is the main immune-activating portion of the molecule. Remarkably, several colistin-resistant mutants of *A.baumannii* have been described with mutations in lipid A biosynthetic genes that eliminate LPS expression

and increase membrane permeability (Moffatt *et al.,* 2010). Finally, the loss of the glycosyltransferase lpsB truncates LPS at the core polysaccharide and revealed that full length LPS were necessary for serum resistance and survival *in vivo* (Luke *et al.,* 2010).

During the invasion, *A. baumannii* will attaches to bronchial epithelial cells (Lee *et al.,* 2008) and following the attachment, *A. baumannii* will invade epithelial cells in a microfilament and microtubule-dependent, zipper-like mechanism (Choi *et al.,* 2008). This interaction will lead to host cell cytotoxicity that specifically, during infection host epithelial cells up regulate the caspase-3, -8, -9, and poly[ADP-ribose] polymerase that correlate with secretion of cytochrome C and apoptosis-inducing factor from the mitochondria (Choi *et al.,* 2005). The stimuli and signalling pathways implicated in cell death are not established; however, they involve imbalanced calcium homeostasis, pro-inflammatory cytokines, and oxidative stress (Smani *et al.,* 2011).

1.3.2 Virulence factors

A. baumannii has a remarkable capacity to survive in unfavourable conditions in virulence mechanisms. This molecular feature promotes environmental persistence; including desiccation resistance, biofilm formation and motility, secretion systems, surface glycoconjugates and micronutrient acquisition systems that facilitate *A. baumannii* pathogenesis (Harding *et al.,* 2018). A study reported that two attributes, drug resistance and environmental persistence, have enabled *A. baumannii* to thrive in the nosocomial environment (Roca *et al.,* 2012).

A. baumannii has the resistancy mechanisms to commonly health-care environments (disinfection, desiccation and oxidative stress) which is similar to antibiotic resistance,

A. baumannii has adapted to those stress (Wong *et al.,* 2017). In desiccation resistance, some of *A. baumannii* clinical isolates are able to maintain viability under dry conditions for almost 100 days (Antunes *et al.,* 2011; Giannoul *et al.,* 2013). The ability of *A. baumannii* to maintain viable under conditions of water limitations is with the help of polysaccharides that are composed of repeating carbohydrate units and function as a glycan shield that encompasses the entire bacterium and protects it from external threats (Scott *et al.,* 2014). The capsular polysaccharide covering *A. baumannii* cells that are grown in a biofilm under dry conditions were likely contributes to the resistance to desiccation in *A. baumannii* (Espinal *et al.,* 2012). Moreover, a study has proposed a relation between the desiccation resistances with the composition of the outer membrane bacteria proteins (Boll *et al.,* 2015). There was also a study on the outer membrane protein A (OmpA) which suggested that *A. baumannii* OmpA contributed to the virulence of the bacteria, in which *A. baumannii* OmpA mutants were shown to be deficient in inducing apoptosis in a human laryngeal epithelial cell line (Choi *et al.,* 2005). The OmpA that localized in the mitochondria will induce apoptosis through the release of the pro-apoptotic molecules cytochrome C and apoptosis-inducing factor, in which might be the pathway of *A. baumannii* inducing the damage to human airway cells during infection. The OmpA protein also plays a role in adherence and invasion of epithelial cells that may contribute to the dissemination of *A. baumannii* during infection (Choi *et al.*, 2008). The OmpA protein also contributes to the ability of *A. baumannii* to persist and grow in human serum as it has been shown that OmpA interacted with soluble inhibitors of the alternative complement pathway and allows the bacteria to avoid complement-mediated killing (Kim *et al.,* 2009). However, OmpA is unlikely to be the only factor that contributes to serum resistance as different *A. baumannii* strains, all of which

contain putative OmpA genes, have significantly different capacities for growth and survival in human serum (Antunes *et al.,* 2011).

Moreover, during periods of desiccation, oxidative stress was also induced. As a result, *A. baumannii* will upregulated proteins that were associated with detoxifying reactive oxygen species (Gayoso *et al.,* 2013). Some strain of *Acinetobacter* are believed to have the highest tolerance to hydrogen peroxide such as strain *A. gyllenbergii* which were able to withstand 100 mM hydrogen peroxide with no loss in viability and maintains viability even in 320mM hydrogen peroxide (Derecho *et al.,* 2014). As *A. gyllenbergii* has been isolated from human specimens, it is likely that more clinically relevant strain such as *A. baumannii* will be more tolerant towards oxidative stressors given (Nemec *et al.,* 2009). In fact, in response to oxidative stress, the emergence of *A. baumannii* strains that contain the insertion sequence element IS*Aba*1 upstream of the catalase gene, *katG*, has been reported, which drives the expression of *katG* and enhances resistance to high levels of hydrogen peroxide (Wright *et al.,* 2017). In hospitals and other health-care settings, disinfectants such as chlorhexidine, are extensively used. These antiseptics are effective against Gram-negative and Gram-positive bacteria which disrupts the cell membranes. But, *A. baumannii* has been shown to actively pump chlorhexidine out of the cell using the *Acinetobacter* chlorhexidine (AceI) efflux protein, thus possibly promoting survival of the bacteria under stressful conditions (Hassan *et al.,* 2013). There were also other disinfectant which is ethanol that has shown to promote the growth and virulence of *A. baumannii* in low concentrations (Nwugo *et al.,* 2012).

Biofilms are encased in an extracellular matrix which likely to have an important role in the interactions of *A. baumannii* with its host and it is also contributes to medical-

device-associated infections. Thompson *et al* reported that during skin and soft-tissue infections, *A. baumannii* populations form robust biofilms (Thompson *et al.,* 2014). It was also reported that *A. baumannii* also forms biofilm communities on most abiotic surfaces, including health-care-associated equipment, such as endotracheal tubes, polycarbonate and stainless steel (Greene *et al.,* 2016a). Greene *et al* also reported that bacteria within biofilm communities, including *A. baumannii*, have increased tolerance to extracellular stresses (Greene *et al.,* 2016b).

1.3.3 Risk factors

Acinetobacter baumannii is an important nosocomial infection in many hospitals, which is difficult to control and treat because of its prolonged environmental survival and its ability to develop resistance to multiple antimicrobial agents (Tseng *et al.,* 2007). *A. baumannii* appear to be an extremely rapid antimicrobial resistance and these resistances are multiple which cause serious therapeutic problems (Cisneros *et al.,* 2002). There are several studies that have been conducted to find the risk factor for bacteraemia caused by multi-drug resistant *A. baumannii* which lead to higher mortality and medical cost compared with non-Multidrug resistance *A. baumannii* bacteraemia. This risk factor might be different between areas with endemic colonization and epidemic outbreak of infection (Shih *et al.,* 2008). From the previous studies of risk factors, it was found that ICU admission, surgical operation, total parental nutrition, invasive procedures such as central nervous 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* (Garcia-Garmendia *et al.,* 2001, Baran *et al.,* 2008). The risk factors that affect individual to *A. baumannii* were similar to those that have been

identified for other multi-drug resistance organisms. Risk factors that are specific for a particular setting have also been identified, such as the hydrotherapy that was used to treat burn patients and the pulsatile lavage treatment that was used for wound treatment (Maragakis *et al.,* 2004). The most frequent clinical manifestations of nosocomial *A. baumannii* are ventilator-associated pneumonia and bloodstream infection which both are associated with considerable morbidity and mortality (Seifert *et al.,* 1995; Cisneros *et al.,* 2002).

1.3.4 Prevalence

The prevalence of nosocomial bloodstream infections due to *A. baumannii* currently has become public health concern in many countries, which contributed from 2% to 10% of all Gram-negative bacterial infections in Europe (Hanberger *et al.,* 1999) and accounted for about 2.5% cases in the United State (Jones *et al.,* 2004). The National Nosocomial Infection Survey data for the US intensive care units indicated that the *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 been increased during this period (Weinstein *et al*., 2005).

It is reported that between 2% and 77% of all *Acinetobacter* clinical isolate species in Asian countries were resistant to imipenem and it was highly prevalent particularly in Thailand and India (Lagamayo, 2008). In Thailand, *Acinetobacter* species caused 44% or 69% of cases of nosocomial pneumonia cases in ICU neonates, and it is reported that 69% of ICU patients with device-associated nosocomial infections was due to infection by ceftazidime-resistant *A baumannii*. Besides, the susceptibility of *A. baumannii* to amikacin was reported to be low, while netilmicin and cefoperazone/sulbactam are the

preferred treatments. In India, *Acinetobacter* species have been found to be high resistance to most antibacterial agents among ICUs patients (Singh *et al.,* 2002). MDR *Acinetobacter* species are very common in India, and it is reported that the use of colistin are many in routine Indian hospitals (Garnacho, 2003). In Korea, 7.4% of all *Acinetobacter* species isolates were resistant to all antimicrobial agents except cefoperazone-sulbactam (15%) and imipenem (13%) and the study described that *Acinetobacter* isolates from sputum were non-susceptible to imipenem (Lee, 2004). In China, a study in 2004 reported that all *A. baumannii* isolates were resistant to multiple antibiotics including meropenem. In that study, 72% to 78% were resistant to the third-generation and fourth-generation cephalosporins (Johnson *et al.,* 1999). While in Singapore, data presented by the researchers indicated that among *Acinetobacter* species, approximately 50% were susceptible to aminoglycosides, yet only approximately 20% were susceptible to carbapenems (Kuah *et al.,* 1994). The study reported that the overall rate of drug resistance in *Acinetobacter* species was 84%, whereas that in VAP cases was reported to be 60% to 70%. In Vietnam, it was reported that the most frequent pneumonia associated to hospital acquired infections (HAIs) was due to *A. baumannii* (Phu *et al.,* 2016). In Malaysia, there is a study on the *A. baumannii* isolated from the intensive care unit (ICU) of a local teaching hospital in Kuala Lumpur from year 2006 to 2009 (Kong *et al.,* 2011). The resistance rate shown by *A. baumannii* to amikacin was decreased from 70.5% in 2006 to 52.0% in 2007 and it was reported increased in prevalence to 91.5% and 97.3% in 2008 and 2009 respectively. The resistance to gentamicin decreased from 91.8% in 2006 to 78.7% in 2008, however, the resistance increased slightly to 83.8% in 2009. Similarly, resistance to trimethoprim/sulfamethoxazole decreased to 88.0% in 2007 and 76.6% in 2008 compared to 98.4% in 2006, then increased slightly to 89.2% in 2009. The clinical

isolates had a high rate of resistance to ampicillin/sulbactam throughout the 4-year period (82.0% in 2006, 88.0% in 2007, 78.7% in 2008, and 94.6% in 2009). Although no cefoperazone/sulbactam-resistance isolates were observed in 2006, resistance was detected in 2007 (40.0%), and the resistance rates increased to 55.3% in 2008 and 62.2% in 2009. All the clinical and environmental isolates of *A. baumannii* were susceptible to polymyxin B.

1.3.5 Antimicrobial susceptibility and resistant mechanisms

Considered susceptible to major antimicrobial drug classes in the 1970s, the clinical importance of the Gram-negative, nosocomial pathogen *A.baumannii* has increased steadily and today this organism is displaying resistance against all first-line antibiotics (Peleg *et al.,* 2008). *A. baumannii* is attracting much attention owing to the increased antimicrobial resistance and occurrence of the strain 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 chloramphenicols (Vila *et al.,* 1993; Seifert *et al.,* 1995). It also has remarkable capacity to acquire mechanisms that confer resistance to broad-spectrum beta-lactams, aminoglycosides, fluoroquinolones and tetracyclines. *A baumannii* exhibited a remarkable ability to rapidly develop antibiotic resistance that led to multidrug resistance within a few decades (Bergogne Berezin *et al.,* 1996).

Some strains of *A. baumannii* have become resistant to almost all currently available antibacterial agents (Looveren,2004; Perez *et al.*,2007) and mostly through acquisition of plasmid (Joshi *et al.,* 2003), transposon (Smith *et al.,* 2003), or integrons carrying cluster of genes encoding resistance to several antibiotic families (Segal *et al.,* 2003;

Poirel *et al.,* 2003). A recent study reported that 58%, 38.7% and 35% of *Acinetobacter* isolates showed resistance to piperacillin-tazobactam, tigecycline and imipenem respectively, and approximately 95% of *Acinetobacter* isolates were MDR, while 12.9% were extended-spectrum β-lactamases (ESBL)-producer (Mishra *et al.,* 2013).

Many studies have suggested the increase trend in *A. baumannii* resistance to majority of the antimicrobial agents. However, because of the scarcity of large-scale surveillance studies 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 depends on biological, epidemiological or methodological factors (Wisplinghoff *et al.,* 2007). It is reported that majority of isolates from the antimicrobial agent tested with one hundred and twenty four *A. baumannii* isolate were determined to be resistant to imipenem (96.9%) in Italy, resistant to colistin were (47.7%) and 34 isolates were susceptible, with an MIC_{50} of 2 mg/L and MIC_{90} of 256 mg/L, isolate from Greece resistance colistin (56.8%) while resistant to tigecycline, five isolates exhibited a resistant phenotype (7.7%), 23 were intermediate (35.4%) and 37 isolates (56.9%) were considered susceptible (Nowak *et al.,* 2017). The resistance of *A. baumannii* to antimicrobial agents is mediated by all of the major resistance mechanism that are known to occur in bacteria, including modification of target sites, enzymatic inactivation, active efflux and decreased influx of drug (Poirel *et al.,* 2003). Beta-lactamase are the most diverse group of enzyme that are associated with resistance, and more than 50 different enzyme, or their allelic forms have been identified so far in *A. baumannii* (Dijkshoorn *et al.,* 2007). In 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* strain through which aminoglycoside resistance can be attributed to at least

nine distinct modifying enzymes with different combination in some strain (Doi *et al.,* 2004). Another study reported that resistance to tetracyclines has been associated with *tet*(A) and *tet*(B) genes that encode tetracycline-specific efflux pump (Huy *et al.,* 2005). ISA*bal* was also thought to have a key role in some carbapenem-resistant strains by enhancing the expression of 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 carbapenemases has also been associated with the modification of penicillin-binding proteins and porins or with up-regulation of the AdeABC efflux system, which might in high-level carbapenems resistance in *A. baumannii* (Bou *et al.,* 2000). Another study showed that production of β-lactamase and reduced expressions of PBP 2 (penicillin-binding protein biotype 2) are the most frequently observed mechanism of resistance to carbapenems (Fernandez-Cenca *et al.,* 2003).

Recently, carbapenem resistance *A. baumannii* isolates in southern Europe which is recovered from patients with VAP were almost universal. Alarmingly, 32% of isolates were multi-drug resistant, 34% were extensively-drug resistant and 31% were pan-drug resistant. From the study also, Rep-PCR has revealed that IC2 as the predominant lineage with 82% of isolates grouping in one large cluster which consisted majority of the PDR isolates. This result suggesting the presence of an epidemic pan-drug resistant *A. baumannii* clone has spread within Greece, Italy and Spain (Nowak, 2017).

1.3.6 Treatment

According to the Infectious Disease Society of America (IDSA), *A. baumannii* is considered as one of the three increasingly problematic Gram-negative pathogens (Daniels, 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 higher than that of control patients who had identical burn severity of illness (Wilson *et al.,* 2004).

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 protein (Levin *et al.,* 2002). Many isolates are now resistant to all aminoglycosides, cephalosporins and fluoroquinolones (Landman *et al.,* 2002). The carbapenems and combination of β-lactam with β-lactamase inhibitor, such as ampicillin-sulbactam, retain useful activity but resistant rates are increasing (Quale *et al.,* 2003). Talbot, (2008) had showed that the carbapenems which have been demonstrated to have greatest inherent activity against *A. baumannii* have increase in resistant frequency from 9% to 39%. While the rate of resistant to fluoroquinolones increased from 50% to 70%. For β-lactam antibiotics, it is increased in resistant from 39% to 66%. All these changes in the epidemiology and resistant rates of *A. baumannii* have led clinicians to adopt therapeutic options, such as colistimethanate sodium (colistin which is also known as polymixin E), the used of which had previously been abandoned in clinical use because of an unacceptable high rate of renal toxicity (Falagas *et al.,* 2005; Li *et al.,* 2006). But, recently, there were some reports of the use of colistin in some pan-drug resistance strain (Roca *et al.,* 2012).

Several studies have tested *in vitro* activity of tigecycline, a semi-synthetic tetracycline (glycycline) against *A. baumannii* and it is reported as good bacteriostatic tetracycline (Curcio *et al.,* 2008). However, there is some doubt in the role of tigecycline in treating MDRAB infection (Ruzin *et al.,* 2007). The ability of *Acinetobacter* to rapidly acquire resistance to this glycycline antimicrobial adds further stimulus for the discovery of newer antimicrobial with activity against this problematic organism (Peleg *et al.,* 2007). Bloodstream infection with *A. baumannii* are occurs with increasing frequency which resulting in significant morbidity and mortality with ranging from 8% to 43% (Scheetz *et al.,* 2007). Thus, clinicians must resort to empirical combination therapy which has an unproven utility where therapeutic failure and relapse is anticipated. A study also have been reported that BAl 30376 (Basilea Pharmaceutica Ltd, Switzerland), a novel β-lactam/β-lactamase inhibitor combination represent an interesting potential approach for MDRAB therapy (Daniel, 2008). However, there is no clinical study has been reported on this therapy.

1.4 The importance of heat shock proteins (HSPs)

Heat-shock proteins (HSPs), or stress proteins, are highly conserved and present in all cells in organisms. Heat shock proteins are present in cells under normal conditions, but are expressed at high levels when exposed to a sudden temperature jump or other stresses (Guimarae *et al.,* 2009). The heat-shock response is the mechanism by which cells react to increases in temperature to prevent damage, and it involves the expression of the almost universally conserved heat-shock genes (Gomes & Simao, 2009). High temperatures and other stresses, such as altered pH and oxygen deprivation, make it more difficult for proteins to form their proper structures and caused some already structured proteins to unfold (Martin *et al.,* 1992), left uncorrected and misfolded

proteins form aggregates that may eventually kill the cell. Heat shock proteins are induced rapidly at high levels to deal with this problem. Nishikawa *et al.,* (2005) reported that in the case of endoplasmic reticulum associated protein degradation C (ERAD-C), degradation is typically dependent on a specific subset of cytosolic chaperones which include Hsp70 and Hsp40 members and this report suggests that these proteins may be directly responsible for recognizing misfolded cytosolic domains of transmembrane proteins. Most heat shock proteins are molecular chaperones. Selected HSPs, also known as chaperones, play crucial roles in folding and unfolding of proteins, assembly of multiprotein complexes, transport and sorting of proteins into correct subcellular compartments, cell-cycle control and signalling, and protection of cells against stress or apoptosis (Martin *et al.,* 1992). Few studies have suggested the role of HSPs in the bacterial stress response caused by antibiotics. Yamaguchi *et al.,* (2003) demonstrated that the chaperones DnaK and GroEL have an effect on the antimicrobial activity of the fluoroquinolone levofloxacin in *E. coli*. According to this study, these chaperones might contribute to quinolone resistance because they sequester the aggregates that accumulate in cells exposed to fluoroquinolones (Guimaraes *et al.,* 2009). Studies have reported that the Hsp70 might inhibit extrinsic and intrinsic pathways of apoptosis by protecting certain proteins such as the oncoproteins Raf-1 and Akt-1, from degradation during elevated temperatures (Davenport *et al.,* 2010; Williamson *et al.,* 2009). Another Hsp70 mechanism was reported to be involved in the cell survival by suppressing the RIP1/3 amyloid formation (Bukau *et al.,* 2006). While another study reported that the Hsp90 are well known to release proteins from chaperone, leading to protein degradation and, ultimately, apoptotic cell death (Matts *et al.,* 2011; Whitesell &, Lindquist, 2005).

1.5 Rationale of the study

The evolution of antimicrobial resistance to multiple antibiotics leads to urgency in developing rapid method for identification of the *A. baumannii* in order to limit and control the outbreak before it can causes serious therapeutics problems. Quick identification of the pathogen in clinical specimens is important to guarantee suitability of clinical treatment and management of the patients. It is also a major difficulty to control the emergence of multidrug-resistance characteristics to the commonly prescribed antibiotics adding the challenge to the treatment of the disease. Multidrug-resistance *A. baumannii* is highly affecting the increase in medical cost, extending time of hospitalization and also shorten life time of patients.

Currently, conventional culture and biochemical methods are being used to detect this organism in majority of clinical medical laboratories. Even though these methods have giving good results, it is time consuming and too laborious for routine diagnostic use. Due to this situation, there is a need in developing an alternative diagnostic method for the early identification of this pathogen in infected patients. The identification of the pathogen at early stage is crucial in order to control the disease outbreak caused by *A. baumannii* and to prevent further complication in infected patients as it has been associated with high mortalities (Nhu *et al.,* 2014).

A study reported that the ability of *A. baumannii* to adapt and to survive in a range of environments has been a key factor for its persistence and success as an opportunistic pathogen (De Silva *et al.,* 2018). As such, HSPs that expressed during high temperatures may have roles in the bacteria to regulate the expression of virulence related proteins. Moreover, the immunogenic properties of HSPs have been widely reported in infectious diseases. Various studies have proven the capability of HSPs as protective vaccines

candidates against bacterial infection (Lee *et al.*, 2006; Wilhem *et al.*, 2005). To date, there is no commercially available kit to detect *A. baumannii* infection. Hence, the antigenic HSP can be a potential biomarker candidate for early detection of this infection. As such, the aim of this study is to identify the presence of HSPs and biomarker(s) in the SAPs of *A. baumannii*. These proteins can be used to develop rapid and reliable protein-based diagnostic tests for the identification of *A. baumannii* which would significantly improve effective management of this infection.

1.6 Objective of the study

Main objective of the study:

To demonstrate presence of specific heat shock protein which is antigenic and specific for *Acinetobacter baumannii*

Specific objectives of this study are as follow:

1. To study the surface associated protein (SAP) profiles of *Acinetobacter baumannii* expressed at 37˚C, 38.5˚C and 41˚C by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
2. To study the immunogenic profile of the SAPs against IgA, IgM and IgG express at 37˚C and 41˚C by Western blot technique
3. To characterize the SAPs of *A. baumannii* that is highly antigenic and specific using mass spectrophotometry.

1.7 Flow chart of the study