PROFILING AND CHARACTERIZATION OF ANTIGENIC SURFACE-ASSOCIATED PROTEINS FOR THE IDENTIFICATION AND DIFFERENTIATION OF *ACINETOBACTER BAUMANNII* FROM NON-BAUMANNII *ACINETOBACTER*

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by

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LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

%	Percentage
+	Plus
®	Trademark
°C	Degree Celsius
µl	Microliter
µM	Micro Molar
µg	Microgram
A260	Absorbance at 260
A280	Absorbance at 280
ACB	*Acinetobacter baumannii* complex
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimeter
dH20	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
e.g.,	Exempli gratia (for example)
et al	Et alia (and others)
F	Forward primer
g	Gram
g	Relative centrifugal force
GC	Guanine-cytosine
h	Hour
HCL	Hydrochloric acid

HRP	Horse-Radish Peroxidase
ICU	Intensive Care Unit
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
k	Kilo
kDa	Kilo Delton
L	Liter
LC-MS/MS	Liquid Chromatography Mass Spectrometry
Ltd	Limited
MALDI-ToF	Matrix-assisted Laser Desorption/Ionization-Time of Flight
MDR	Multi-Drug Resistant
mg	Milligram
ml	Milliliter
mm	millimeter
MW	Molecular weight
n	Nano
n	Nanometer
NI	Nosocomial Infections
NCBI	National Center for Biotechnology Information
OD	Optical density
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate buffer Saline
PCR	Polymerase Chain Reaction
R	Reverse primer
RNA	Ribonucleic acid

rpm	Revolution per minute
rpoB	RNA Polymerase B
rRNA	Ribosomal RNA
SAP	Surface-Associated Proteins
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
sp.	Species (singular)
spp.	Species (plural)
Ta	Annealing temperature
Tm	Melting temperature
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris Hydrochloride
™	Trademark
U	Unit
USA	United State of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volt
v	Volume
v/v	Volume/volume
VAP	Ventilator-Associated Pneumonia
w/v	Weight/volume

LIST OF APPENDICES

PENENTUAN PROFIL DAN IDENTIFIKASI PROTEIN PERMUKAAN ANTIGENIK UNTUK PENGENAL PASTIAN *ACINETOBACTER BAUMANNII* DAN PEMBEZAAN *A. BAUMANNII* DARIPADA *ACINETOBACTER* BUKAN BAUMANNII

ABSTRAK

Jangkitan nosokomial yang disebabkan oleh *Acinetobacter* merupakan masalah kesihatan awam sejagat. Tempoh diagnosis jangkitan nosokomial *Acinetobacter* yang panjang serta kadar kematian yang berkaitan dengannya telah menjadi kelemahan utama dalam pengurusan pesakit. Oleh itu, ujian pengesanan yang pantas dan boleh dipercayai serta mampu membezakan *Acinetobacter baumannii* daripada *Acinetobacter nosocomialis* dan *Acinetobacter pittii* adalah perlu. Protein permukaan sel (SAPs) telah dikenalpasti potensinya dalam bertindak sebagai penanda biologi yang boleh digunapakai untuk mengesan jangkitan bakteria lain seperti *Salmonella*. Sehubungan dengan itu, kajian ini bertujuan untuk menentukan profil SAPs bagi *A. baumannii*, *A. nosocomialis* dan *A. pittii* serta penentuan ciri-ciri antigenik SAPs tersebut dengan menggunakan teknik blot Western. Ini bertujuan mengenalpasti calon-calon SAPs yang berpotensi digunakan dalam pembangunan ujian pengesanan pantas yang menggunakan serum pesakit terinfeksi dengan *A. baumannii*, *A. nosocomialis* dan *A. pittii*, termasuk juga serum yang positif terhadap jangkitan selain *Acinetobacter*. Keputusan kajian ini menunjukkan bahawa 46.4% daripada jaluran profil SDS-PAGE SAP tersebut merupakan protein yang umum bagi ketiga-tiga spesis *A. baumannii, A. nosocomialis* dan *A. pittii*. Manakala, kajian lanjutan profil SDS-PAGE SAP menunjukkan bahawa terdapat tiga jaluran SAP bersaiz molekul 34.4 kDa, 36.0 kDa dan 40.9 kDa yang unik bagi *A. baumannii*, dua

jaluran bersaiz 33.0 kDa dan 55.1 kDa yang unik bagi *A. nosocomialis*, dan dua jaluran protein bersaiz 35.0 kDa dan 43.0 kDa yang unik bagi *A. pittii*. Analisis teknik blot Western ke atas profil SAPs menunjukkan bahawa terdapat variasi dalam tindak balas imun perumah terhadap protein-protein SAP tersebut. Bagi strain *A. baumannii* AB1001, kajian antigenisiti mendapati sebanyak 86.4% daripada protein SAPs adalah bersifat antigenik, yang mana IgG dan IgM dikesan dengan kadar yang tinggi iaitu masing-masing sebanyak 84.2%, diikuti dengan IgA (57.9%). Bagi strain *A. nosocomialis* AN1001, 70% daripada profil SAP tersebut bersifat antigenik dengan pengesanan IgM sebanyak 100%, diikuti dengan IgG (92.9%) dan IgA (57.1%). Manakala, 90% daripada profil SAP strain *A. pittii* AP1001 adalah antigenik dengan pengesanan tertinggi adalah bagi IgG (88.8%), diikuti oleh IgA (77.8%) dan IgM (61.1%). Hasil daripada analisis tersebut telah mengenalpasti beberapa protein SAP khusus bagi *A. baumannii* (40.9 dan 34.4 kDa), *A. nosocomialis* (33.0 dan 55.1 kDa) dan A. *pittii* (43.0 dan 35.0 kDa) yang dikenal pasti oleh IgG, IgM, dan IgA. Pencirian protein SAP menggunakan kaedah spektrometri LC-MS/MS mengesahkan bahawa SAP khusus bagi *A. baumannii* yang bersaiz 34.4 kDa, 40.9 kDa, 48.7 kDa dan 23.0 kDa masing-masing adalah sebagai OmpA, Omp38, protein faktor pemanjangan dan protein faktor kitar semula ribosom. Sementara itu, SAPs bersaiz 23.0 kDa, 33.0 kDa, 48.7 kDa dan 55.1 kDa daripada *A. nosocomialis* masing-masing dikenal pasti sebagai protein 50S ribosomal L4, protein ribosomal 30S, protein beta subunit ATP synthetase, dan protein chaperonin. SAPs bersaiz 35.0 kDa dan 43.0 kDa daripada *A. pittii* pula masing-masing dikenal pasti sebagai Omp38 dan faktor pemanjangan *Acinetobacter*. Pengenalpastian SAPs antigenik yang khusus bagi spesis tertentu dalam *Acinetobacter* kompleks, serta SAPs antigenik yang umum bagi semua ahli *Acinetobacter* kompleks memberi maklumat yang penting ke arah pembangunan ujian pengesanan pantas

jangkitan *Acinetobacter*. Ini membolehkan diagnosis awal dilakukan serta dapat membezakan jangkitan *A. baumannii* daripada jangkitan bakteria lain seperti *A. nosocomialis* dan *A. pittii*. Walau bagaimanapun, kajian penilaian lanjutan mengenai SAPs khusus tersebut perlu dijalankan secara *in vivo* supaya potensi diagnostik protein ini dapat dinilai dengan lebih mendalam. Protein penanda yang telah dikenal pasti dalam kajian ini boleh dikembangkan lagi penggunaannya ke arah pembangunan ujian pantas seperti ujian dipstik, kaedah immunoasai enzim atau ujian molekular lain yang dapat membantu dalam pengesanan jangkitan *Acinetobacter*.

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ABSTRACT

Acinetobacter nosocomial infections is a universal public health care problem. The time taken to diagnose *Acinetobacter* nosocomial infections and the rate associated mortality is a major drawback in patient management. Members of the ACB complex differ in their biology and antibiotic susceptibility pattern, which significantly impede the proper and prompt treantment of the patient. Hence, there is a need for a rapid and reliable identification test that not only rapidly identify *Acinetobacter* but can differentiate *A. baumannii* from *A. nosocomialis* and *A. pittii*. Surface-associated proteins (SAPs) proved to be reliable for such task in other bacteria such as *Salmonella*. *Acinetobacter* contain in their outer membrane lipopolysaccharides (LPS), which represent useful diagnostic markers. Characterized LPS of *Acinetobacter* have been shown to be of the smooth (S)-form. Therefore, a possible O-serotyping scheme represents a powerful tool in clinical laboratories to identify *Acinetobacter* species. The aim of this study was to profile the SAPs of *A. baumannii, A. nosocomialis* and *A. pittii* and to determine the antigenicity of the SAPs by Western blot towards identification of potential candidate for the development of rapid diagnostic test using serum from patients infected with *A. baumannii, A. nosocomialis* and *A. pittii* as well sera positive with other non-*Acinetobacter* infections. The SAPs of *A. baumannii, A. nosocomialis* and *A. pittii* was profiled using SDS-PAGE, antigenicity of the profiled SAPs was determined by Western Blot techniques.

Exclusive SAPs to each of the three species, as well as those SAPs common to the three species (ACB complex) were characterized by LC-MS/MS technique. Result of SDS-PAGE profiles in this study demonstrated that 46.4% of the SAP bands were common to *A. baumannii*, *A. nosocomialis,* and *A. pittii*. The SDS-PAGE SAPs profiling further unveiled the presence of SAP bands (40.9, 36.0, and 34.4 kDa), which are unique to *A. baumannii*, 55.1 kDa and 33.0 kDa unique to *A. nosocomialis*, and 43.0 kDa and 35.0 kDa unique to *A. pittii*. Western blot analysis of the profiled SAPs showed variation in host immune response in all the sera tested. In *A. baumannii* AB1001, the antigenicity study revealed that 86.4% of the SAPs from SDS-PAGE profiles were antigenic. IgG and IgM detected a higher percentage of 84.2% each, followed by IgA 57.9%. In *A. nosocomialis* AN1001, 70% of the SAPs profiles from SDS-PAGE were antigenic, of which IgM detected all 100%, IgG ranked the second 92.9%, and then IgA 57.1%. Likewise, in *A. pittii* AP1001, 90% of the SAPs profiles from SDS-PAGE were antigenic. Among them, IgG detected a higher number (88.8%), IgA (77.8%), and IgM (61.1%). Furthermore, the *A. baumannii, A. nosocomialis* and A. *pittii* exclusive SAPs (40.9 and 34.4 kDa; 33.0 and 55.1 kDa and A. *pittii* 43.0 kDa and 35.0 kDa respectively) were recognised by the IgG, IgM, and IgA. The LC-MS/MS identification of the selected SAPs confirmed that the 34.4, 40.9, 48.7 and 23.0 kDa from *A. baumannii* were identified as OmpA, Omp38, an elongation factor protein and ribosome-recycling factor protein respectively. Whereas the SAPs of 33.0, 48.7, 55.1, and 23.0 kDa from *A. nosocomialis* were identified as 30S ribosomal protein, ATP synthetase subunit beta protein, chaperonin protein and 50S ribosomal L4. While the 35.0 kDa and 43.0 kDa from *A. pittii* were identified as Omp38 and *Acinetobacter* elongation factor respectively. The identification of antigenic SAPs exclusive to each member of the ACB complex and antigenic SAPs

common to all members of the ACB complex could pave way in the development of rapid diagnostic test for the early diagnosis of *Acinetobacter* infection and differentiation of *A. baumannii* from *A. nosocomialis* and *A. pittii* infection. However, further studies on the species exclusive SAPs need to be carried out in vivo to evaluate the diagnostic performance of these proteins. Therefore, the biomarkers identified in this study can be further developed in the form of rapid test such as dipstick test, enzyme immunoassay or molecular assay, which can be very helpful in diagnosing patients with *Acinetobacter* infection.

CHAPTER 1

INTRODUCTION

1.1 History and nomenclature of the genus *Acinetobacter*

The genus *Acinetobacter* is composed of a complex and heterogeneous group of bacteria that inhabit many natural habitats and have a diverse ecological and clinical significance (Anane *et al*., 2019; Hrenovic *et al*., 2014). *Acinetobacter* belongs to the family *Moraxellaceae*, which encompasses bacterial genera *Moraxella, Psychrobacter*, and some closely related bacteria (Rossau *et al*., 1991). *Moraxellaceae* family belongs to the order *Pseudomonadales* of the class *Gammaproteobacteria* (Rossau *et al*., 1991).

The genus *Acinetobacter* has been assigned with different names ever since its discovery in 1911. A Dutch Microbiologist, Martinus Willem Beijerinck, was the first to report the organism and named it *Micrococcus calcoaceticus* (Beijerinck, 1911). From 1911 to 1970, the genus *Acinetobacter* acquired different names. However, most turned out to belong to the same genus *Acinetobacter*. The different names include *Achromobacter anitratum, Achromobacter mucosus, Alcaligenes haemolysans, Bacterium anitratum, Diplococcus mucosus, Herellea vaginicola, Micrococcus calcoaceticus, Mima polymorpha, Moraxella lwoffii, Moraxella lwoffii var. glucidolytica,* and *Neisseria winogradskyi* (Almasaudi, 2018; Baumann, 1968). These bacteria shared several biological features until, in 1968, they were grouped under the current genus name, *Acinetobacter*, following a comprehensive review of their phenotypic characteristics (Baumann, 1968). Consequently, the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria in 1971 recommended the official acknowledgment of the genus *Acinetobacter* (Lessel, 1971). Coincidently, the index

clinical isolate of *Acinetobacter* was isolated from the Intensive Care Unit (ICU) around that time, in 1969.

The advent of DNA-DNA hybridisation molecular techniques in 1986 led to 12 new species in the genus *Acinetobacter* that showed more than 70% DNA-DNA similarity. The added species are *Acinetobacter haemolyticus, Acinetobacter jonhsonnii, Acinetobacter junnii,* and *Acinetobacter lwoffii* (Bouvet and Grimont, 1986). However, some of the *Acinetobacter* species were not assigned names at that time. A few years later, another 31 genomic species were registered in the genus, 17 assigned with species names. The remaining are called "Genomic Species" with a number attached to them (Bouvet & Jeanjean, 1989; Nemec *et al*., 2003; Tjernberg *et al*., 1989).

Following tremendous progress in molecular identification techniques in 2012, the number of *Acinetobacter* genomic species identified increased to 36, with 27 validly named species (Peleg *et al*., 2012). Identification of novel *Acinetobacter* species is still ongoing, in addition to the renaming of the previously described genomic species following extensive phenotypic and genotypic characterization (Nemec *et al*., 2015). The genus *Acinetobacter* presently consists of about sixty-three (63) validly published species, ten (10) effectively named but not published species, and 17 tentatively nominated species (http://apps.szu.cz/anemec/ Classification.pdf), the majority are none pathogenic, and more species are expected to be discovered.

Another revelation made by the DNA-DNA hybridizationl technique is that, within the genus *Acinetobacter*, the species *Acinetobacter calcoaceticus, Acinetobacter baumannii*, Genomic Species 3, and Genomic Species 13TU are highly related to each other as compared to the rest of the members of the genus (Bouvet &

Grimont, 1986; Tjernberg *et al*., 1989; Vos *et al*., 2016). Consequently, these four species are later grouped to bear a single name, the *Acinetobacter calcoaceticus–A. baumannii* (ACB) complex, a name first proposed by Gerner-Smidt *et al*. (1991). Advanced molecular analysis of bacterial housekeeping genes (Périchon *et al*., 2014) and whole-genome analysis (Touchon *et al*., 2014) later confirmed the relatedness of these species. Furthermore, using the same techniques, Genomic Species 3 was confirmed to be *Acinetobacter pittii,* while Genomic Species 13TU was renamed as *Acinetobacter nosocomialis* (Nemec *et al*., 2011).

In a continuous advancement in the identification and nomenclature of *Acinetobacter* species, two more Genomic Species were included in the group ACB complex. Previously these Genomic Species were reported as "Close to 13TU" and "Between 1 and 3" because of their similarities. Recently molecular characterization study confirmed Genomic Species "Close to 13TU" as *Acinetobacter seiferttii* (Nemec *et al*., 2015) " and "Between 1 and 3" to be *A. dijkshooniae* (Marí-Almirall *et al.*, 2016). Routine biochemical tests cannot distinguish members of the ACB complex from another. Consequently, infections caused by members of this complex are commonly reported as *Acinetobacter calcoaceticus-A. baumannii* (ACB) complex infections. Among the ACB complex, *A. baumannii* is frequently implicated in nosocomial infections. However, in clinical-related publications, *A. nosocomialis and A. pittii* are increasingly surfacing as causative agents of nosocomial infections (Fernández-Cuenca *et al*., 2019).

1.2 Reservoirs of *Acinetobacter* species

Acinetobacter species are ubiquitous (found in soil, water, human and animal bodies (Houang *et al*., 2001) and harsh environments such as hot springs (Goic-barisic *et al*., 2016). Some genus members, such as *A. calcoaceticus*, exhibit a mutual relationship with plant roots, signifying its active involvement in the ecosystem's nutrient cycle (Pontiroli *et al*., 2009). The genetic determinant exchange has also been documented between *A. baylyi* and plant roots via mobile genetic elements (Pontiroli *et al*., 2009).

As far back as 1997, the healthy human individual's body has been reported to house *Acinetobacter* species. This was evidenced by the frequent isolation of a significant percentage of *Acinetobacter* species from human clinical specimens (Peleg *et al*., 2008). The commonly isolated species from human clinical specimens comprises *A. lwoffii, A. jonhsonnii, A. junii, A. baumannii, A. nosocomialis,* and *A. pittii*. They colonise the body's moist secretory regions: burned skin, the mucosal lining of the respiratory and urinary tracks (Dijkshoorn *et al*., 2007).

From the patient, hospital environment, and equipment surfaces, the frequently isolated species is *A. baumannii* (Peleg *et al*., 2008). *A. baumannii* not only dwell between the hospital setting and hospital staff and their family (Pogue *et al*., 2014), recently *A. baumannii* was reported to have colonised and infected immunocompromised animals in veterinary ICU (Zordan *et al*., 2011).

1.3 Physical and chemical properties of *Acinetobacter* species

Clinically relevant *Acinetobacter* species are reported to be resistant to chlorhexidine, gluconates, and phenol-derivatives, and radiation (Roca *et al*., 2012). This could be why ordinary sterilisation of the hospital equipment cannot effectively remove *Acinetobacter*; however, sterilisation by ethylene oxide can effectively clear *Acinetobacter* (Abbo *et al*., 2005; Houang *et al.*, 1998). The *A. baumannii* and *A. lwoffii* can persist in the clinical environment and hospital equipment surfaces even with desiccation for up to a month compared with other *Acinetobacter* species such as *A. jonhsonnii* (Barbolla *et al*., 2003). *A. radioresistens* have been reported to persist in an environment for almost five and a half months (Roca *et al*., 2012). *Acinetobacter* can survive desiccation more than *E. coli* (Barbolla *et al.*, 2003).

1.4 Morphological and cultural characteristics of *Acinetobacter*

Morphological and cultural traits have been useful features for the identification of bacteria (Patel, 2013). The genus *Acinetobacter* consists of Gramnegative, non-fermentative, strictly aerobic, nonmotile bacteria. They are catalasepositive, indole-negative, oxidase-negative, encapsulated coccobacillus rods with a G+C content of 39-47%.

Acinetobacter species are non-fastidious and can be easily grown on traditional laboratory media at 20-30°C, with the clinical isolates growing at 37 to 44°C. In the exponential phase of growth, they appear as bacilli 0.9 to 1.6 μm in diameter and 1.5 to 2.5 μm in length, often in pairs or assembled into longer chains of varying size (Antunes *et al*., 2011; Bouvet & Jeanjean, 1989; Peleg *et al*., 2008). On blood agar plates, colonies display typical shapes and sizes, being colorless (white or creamcolored), smooth, or mucoid (when the capsule is present), milky, 1-2 mm in

diameter (after 18–24 h incubation at 37°C). In contrast, colonies display bluish to bluish gray colours on eosin methylene blue agar. On Herellea agar, the colonies are pale lavender, while on Leeds *Acinetobacter* medium, pink mucoid colonies with pink color diffused into the medium (Peleg *et al*., 2008)

The ACB-complex shows cultural characteristics like Enterobacteriaceae on solid media, forming a wide range of colony types. Other *Acinetobacter* species produce tiny glowing colonies with no hemolysis on blood agar, yet some species may show hemolysis on 5% sheep blood agar.

1.5 Statement of research problems and justification of the study

Acinetobacter species appeared to be topmost nosocomial pathogens troubling mostly patients with weakened host defence system in intensive care units and are accountable for several hospital outbreaks of bacterial nosocomial infections (LópezDurán *et al.,* 2020). The ACB complex has repeatedly been reported to cause nosocomial infection (Nafarieh *et al.,* 2017; Wpob1zbi8=0einstein *et al*., 2005; Wisplinghoff *et al*., 2000). Substantial number of *Acinetobacter* surface proteins antigenicity studies focused on *Acinetobacter baumannii*, with most of the studies been devoted to the roles of OMPs, whereas the SAPs have attracted less attention (Latasa *et al*., 2006). Compared with the close relatives of *A. baumannii* such as *A. nosocomialis* and *A. pittii*, less is known regarding their SAPs antigenicity.

The time taken (5 to 7 days) by the culture and automated methods in the diagnosis of *Acinetobacter* nosocomial infections is an increasing concern due to the increase mortality rate associated with the delay. It was estimated that *Acinetobacter* BSI are associated with high mortality rates (17-52%), and the time taken to produce laboratory diagnosis result contributed to the hike in mortality rate (Cisneros *et al,*

1996; Wisplinghoff *et al*, 2000; Zhou *et al*., 2019). These emphasize the importance of early diagnosis of the *Acinetobacter* infection to enable effective therapy and management of the disease (Vrancianu *et al.*, 2020).

Serologic tests demonstrated good option for the rapid diagnosis of many bacterial diseases (Tuuminen *et al*., 2013). Antibodies agaist invading bacterial pathogen begin to appear in the blood in 7 to 10 days following an infection. The used of serum samples from patient infected with *Salmonella* proved to useful for the rapid diagnosis of typhoid fever (Muralinath *et al*., 2011). Currently, the focus has shifted towards proteins as diagnostic candidate, chiefly targeting bacterial surface proteins, because they are more exposed and thus, more accessible to antibodies. Similar to other gram negative bacteria (Geisinger *et al*., 2019), *Acinetobacter* contains lipopolysaccharide (LPS) on the surface of its outer membrane. Since LPS has been used successfully as antigenic markers for many bacteria such as *Salmonella* Typhi for the development of serodiagnostic tests (Ismail, 2000; Muralinath *et al*., 2011). This present study explores the surface membrane protein(s) of *Acinetobacter* with the aim to provide a proteomic basis for the identification *Acinetobacter baumannii* and differentiation of *A. baumannii* from *A. nosocomialis* and *A. pittii* which may be helpful for the development of rapid diagnostic test.

1.6 General objective of the study

To identify antigenic surface-associated proteins that could be used for the identification and differentiation of *Acinetobacter baumannii* from *A. nosocomialis* and *A. pittii*.

1.6.1 Specific objectives of the study

i. To profile pan-surface-associated proteome of *A. baumannii, A. nosocomialis* and *A. pittii* by SDS-PAGE.

ii. To determine the antigenicity of the surface-associated proteins of *A. baumannii, A. nosocomialis* and *A. pittii* by Western blot.

iii. To identify surface-associated proteins exclusive to *A. baumannii, A. nosocomialis* and *A. pittii*, and surface-associated proteins common to all the three species.

iv. To characterize the selected antigenic SAPs of *A. baumannii, A. nosocomialis* and *A. pittii* by liquid chromatography tandem mass spectrometry (LC-MS/MS).

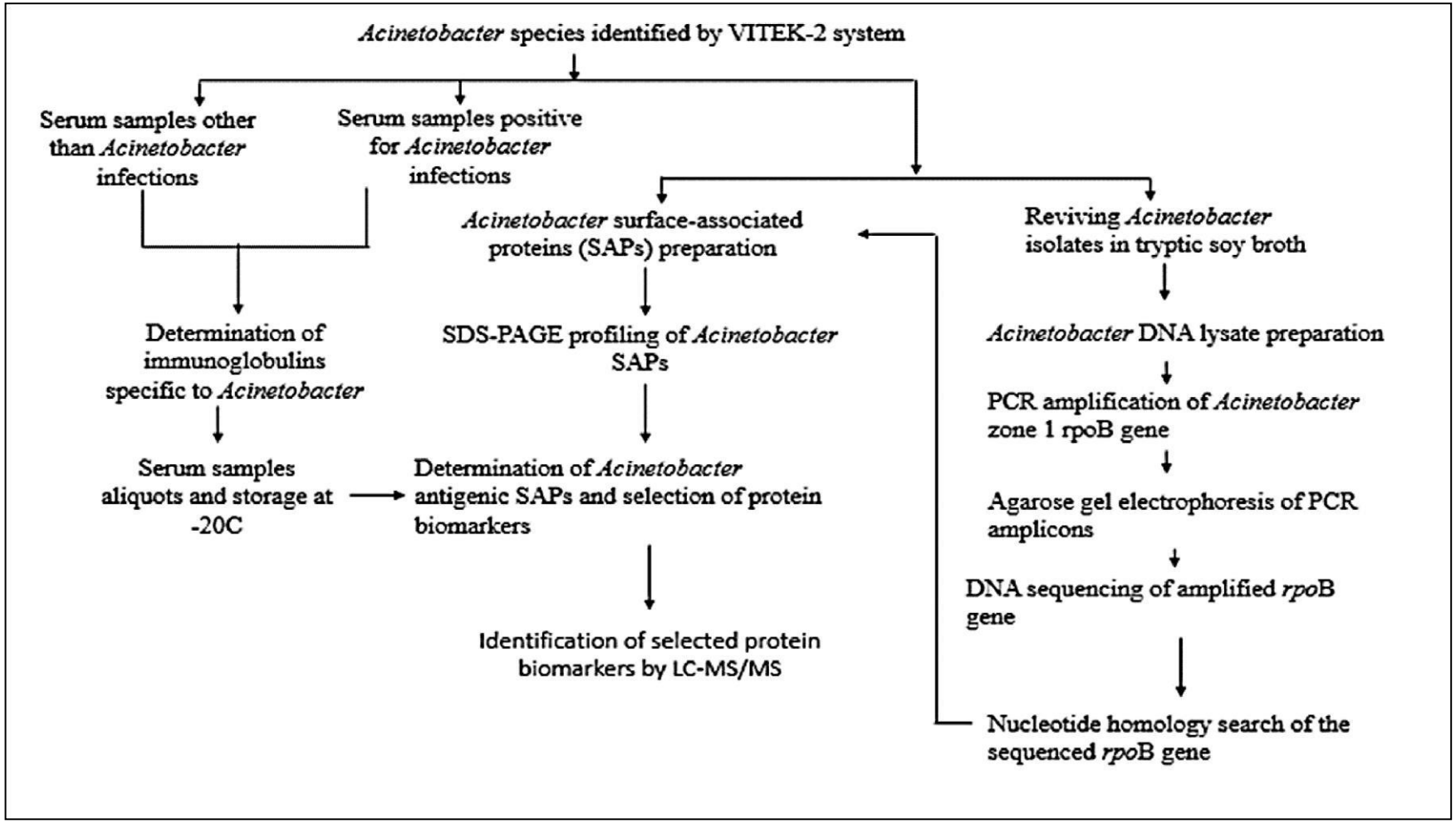

Figure 1.1 Research flowchart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 *Acinetobacter* species cell surface proteins

There is a growing concern about the delay (5 to 7 days) in the detection of *Acinetobacter* nosocomial infections by the currently used culture and automated identification techniques (VITEK 2 System and MALDI-ToF MS) in clinical laboratories and the high mortality rate associated with the delay (Esther *et al*., 2018). Inappropriate empiric treatment in patient with HAIs due to *Acinetobacter* is associated with 80% upsurge in hospital mortality and high cost of hospital treatment (Zilberberg *et al.,* 2017). A study from Thailand in an attempt to assess the effects of the delay in the detection of *Acinetobacter* nosocomial infection and its associated mortality rate revealed an increase in mortality from 33.8% to 40.4% (Lim *et al*, 2021). These characteristics have led to a pressing need to explore alternative ways for rapid diagnoses, such as bacterial surface-associated proteins. The *Acinetobacter* cell surface structures play essential roles in the interactions with the host and stimulation of the immune response against foreign substances (Silhavy *et al*., 2019). The molecular structures present on the cell surface, and those that extend beyond the surface, may be of importance for the diagnosis of the ACB complex; these are often the key determinants that mediate bacterial virulence and thus represent important targets for novel diagnostic biomarkers (Grandi, 2010). The followings are the highlights of the *Acinetobacter* surface proteins, which may be used as biomarker candidates for the development of a rapid diagnostic test for Acinetobacter nosocomial infections.

2.1.1 Glycoproteins

The addition of sugar moieties to proteins (posttranslational modification), in bacteria occurs via the amide of asparagine residues (*N*-linked) or the hydroxyl of serine/threonine residues (*O* linked). In a sequences of steps comparable to O antigen biosynthesis, sugar moieties are assembled onto the Und-P lipid carrier and moved to the periplasmic space of the inner membrane, where an *O*-oligosaccharyltransferase (*O* OTase) enzyme catalyzes the transfer of the complete carbohydrate structure to a serine or threonine residue on the cognate acceptor protein (Moran, 2007). Bioinformatic investigation confirmed a protein in *Acinetobacter baumannii* that is similar to the *O*-OTase from *Neisseria meningitidis*, as *PglL* (Moran, 2007). The PglL proteins often carries domains analogous to those of WaaL ligases (Wzy C domains), and bioinformatic confirmation of a PglL-like protein is not adequate to distinguish it from WaaL ligases, thus demanding experimental characterization.

The SDS-PAGE analysis of *A. baumannii* devoid of *pglL* gene showed the absence of carbohydrate-specific band, with no change in the LOS profile (Moriel *et al*., 2013). Further characterization of the *A. baumannii pglL* mutant by mass spectrometry confirmed that the *pglL*-deficient strain to be short of seven glycoproteins, which were conjugated with five sugar moieties in the wild-type strain. Lack of protein glycosylation in *A. baumannii* led to pleiotropic effects on numerous virulence-associated phenotypes, including biofilm formation and survival in a mouse model of systemic infection (Mcqueary *et al*., 2012). Protein glycosylation is phenomenon observed throughout the genus *Acinetobacter*, but then the configuration of the glycan moiety and the number and distinctiveness of the modified proteins vary among strains (Campanero-Rhodes *et al*., 2020). The carbohydrate structure attached

to glycoproteins is similar to the repeat units found in capsular polysaccharide (Kalynych *et al.,* 2014).

In the *A. baumannii* the *pglL* gene is located downstream of the type IV pilin gene, *pilA*, which encodes for glycosylation also in bacteria. In laboratory study this protein was not found to be glycosylated in *A. baumannii* ATCC 17978 but was reported to be glycosylated upon overexpression in the presence of PglL (Yang *et al*., 2019). Most *Acinetobacter* spp. possessed two proteins with a domain from the Wzy_C superfamily that is common to PglL and WaaL orthologs.

2.1.2 Capsule

Acinetobacter is not an exception like other bacteria, *Acinetobacter* spp. also produce an extracellular capsular layer that serves a layer of protection from external threats such as macrophages (Ramirez *et al*., 2019). The capsular layer production and protein glycosylation are finely linked in *Acinetobacter*, as the carbohydrate repeat unit found in the capsular layer is identical to the single repeat unit linked to proteins (Yang *et al*., 2017). It was recently described that capsule production may perhaps be increased due to the subinhibitory levels of antibiotics, which increased resistance to Phagocytes and led to a hypervirulent phenotype in a mouse model of systemic infection (Fernando & Kumar, 2013). This capsule hyperproduction phenotype was shown to be controlled by the two-component BfmRS system, which regulates several other important virulence factors in *Acinetobacter* (Schramm *et al.,* 2019). *Acinetobacter* spp. also produce a surface-associated poly--1-6-*N*-acetylglucosamine (PNAG) polysaccharide, which is important for virulence and biofilm formation (Luo, *et al.,* 2020).

2.1.3 Pili

Bacteria surface appendages, such as pili, mediate contacts between the bacteria and their environment. *Acinetobacter* pili was first reported by Henrichsen and Blom in 1975, that *Acinetobacter calcoaceticus* strains exhibiting surface fimbrial structures showing twitching motility (Na *et al*., 2015), a form of bacterial motion recent researches showed that it dependent on functioning type IV pili (Latasa *et al*., 2006). Furthermore, studies also revealed that the nonpathogenic *Acinetobacter baylyi* ADP1 produces both thin and thick pili (Pontiroli *et al*., 2009). A collection of chaperone/usher pili, referred to as Csu pili, has been showed to be conserved in all sequenced pathogenic *Acinetobacter* spp.; however, the Csu pili have been specifically studied in *A. baumannii* (Kalynych *et al*., 2014). The *Acinetobacter* Csu pili are essential for the production and functions of biofilm in *A. baumannii* ATCC 19606 but not needed for the attachment to human epithelial cells (Piepenbrink *et al*., 2016). Additional study also reported that the CsuA/B pilin subunit was the chief protein within the pellicle matrix of multiple *A. baumannii* strains (Mayasari & Siregar, 2014), further suggesting the role of Csu pili in biofilm formation and maintenance.

Previous studies also revealed a single nucleotide insertion in the *csuB* gene of *A. baumannii* ATCC 17978, signifying that this system may be non-essential in this strain (Lopes *et al*., 2017); however, recent resequencing of the *A. baumannii* ATCC 17978 genome (GenBank accession no. CP012004) did not find the same insertion event (D'Souza *et al*., 2019).*Acinetobacter* spp. of clinical importance have also been shown to produce type IV pili (Tfp), which are dynamic bacterial surface appendages known to mediate twitching motility, horizontal gene transfer, and biofilm formation (Ripoll-rozada *et al*., 2015). Although bioinformatic studies have confirmed the existence of genes thought to encode for proteins needed for the biogenesis of Tfp in

A. baumannii, only *Acinetobacter nosocomialis* strain M2 has been shown to produce functioning Tfp (Niu *et al*., 2008), which is glycosylated by a TfpO-like oligosaccharyltransferase (Luke *et al*., 2010). Several *A. baumannii* strains have been found to be naturally transformable and showing twitching motility (Schramm et al., 2019), two classic Tfp-associated phenotypes, which strongly indicate their presence. Tfp-like structures were also identified on *A. baumannii* ATCC 17978 (Bazmara *et al*., 2019); furthermore, mutants in predicted Tfp biogenesis components of *A. baumannii* ATCC 17978 exhibited impaired biofilm formation (Ambrosi *et al*., 2017) but the major pilin subunit, PilA, has not been shown to be surface exposed and/or associated with the pilin structures observed. Although Tfp, with roles in *Acinetobacter* motility and natural transformation, has emerged as a possible virulence factor, no studies have conclusively linked Tfp to the pathobiology of *Acinetobacter*, as is the case for *Pseudomonas* and *Neisseria*.

2.1.4 Type II secretion

The most recently described secretion system is a functional type II secretion system (T2SS) identified in both *A. nosocomialis* strain M2 (Nho *et al.*, 2015) and *A. baumannii* ATCC 17978; moreover, it was shown that clinical isolates of *Acinetobacter pittii*, *A. baumannii*, *A. calcoaceticus*, and *Acinetobacter junii* all were able to secrete type II substrates, indicating that functioning type II secretion systems seem to be the rule and not the exception. With regard to the T2SS of *A. nosocomialis* strain M2, a two-dimensional differential gel electrophoresis approach identified multiple putative type II substrates; the LipA and LipH lipases and the CpaA metallopeptidase were validated as *bona fide* type II secretion substrates.

Interestingly, both LipA and CpaA required specific membrane-associated chaperones for secretion, which indicates that T2SS chaperones are more widespread than previously recognized. Importantly, it was shown that an *A. nosocomialis* strain M2 *gspD* mutant lacking the outer membrane secretin of the T2SS was severely attenuated in both the *Galleria mellonella* and murine pulmonary infection models. Specifically, mice intranasally infected with the *gspD* mutant strain had 2-log lower bacterial burdens in both the lungs and the spleen after 36 h, compared to both the parental strain and the complemented mutant. In *A. baumannii* ATCC 17978, Johnson et al. identified a lipase, LipA, secreted in a T2SSdependent manner that was required for growth on medium containing lipids as a sole carbon source (Putker *et al.*, 2015). Mutants with mutations in *lipA* or the T2SS structural gene *gspD* were less competitive than the wild-type strain in a mixed-infection murine model of bacteremia. Collectively, these findings indicate that the *Acinetobacter* T2SS is a previously unrecognized virulence factor mediating pathogenesis in a relevant mammalian model. Interestingly, a recent study by Wang *et al.*, utilized an *A. baumannii* ATCC 17978 *gspN* mutant for validation of their insertion sequencing murine pulmonary infection studies, and they subsequently found that the *gspN* mutant did not display any virulence defect in survival or competition models, compared to the parent strain (Diepold & Wagner, 2014). Although these data are in contrast to the newly defined role of type II secretion in *Acinetobacter*, it was demonstrated previously that *gspN* homologs were not required for a functioning T2SS in *Klebsiella oxytoca* (Weber *et al*., 2017); furthermore, *gspN* homologs are absent from numerous known T2SSs in other Gram negative bacteria (Suzuki *et al.*, 2015), indicating the dispensable nature of GspN in functioning T2SSs.

2.1.5 Autotransporters

A type V autotransporter has been characterized in *A. baumannii*. The *Acinetobacter* trimeric autotransporter (Ata) was found to be crucial for the ability of certain *A. baumannii* strains to adhere to extracellular matrix components, including collagen I, III, IV, and V (Eijkelkamp *et al*., 2011). Ata is also an important mediator of *A. baumannii* biofilm formation and maintenance, as an *A. baumannii* ATCC 17978 *ata* mutant had significantly diminished biofilm production and was less virulent in a murine intraperitoneal infection model, compared to the parental and complemented strains (Ramezanalizadeh *et al*., 2020).

2.1.6 Type VI secretion

Bacteria interact with each other in a multitude of ways; these interactions are often competitive in nature and play important roles in niche establishment (Ackerveken *et al.,* 2016). The bacterial type VI secretion system (T6SS) was first formally described for *Vibrio cholerae* and *Pseudomonas aeruginosa,* and was suggested to play a role against eukaryotic hosts (Gurung *et al*., 2013). While several Type VI Secretory Systems have been determined to secrete anti-eukaryotic toxins, it has recently been appreciated that many bacteria use their T6SSs to secrete antibacterial toxins to kill competing bacteria (Li *et al*., 2019).

The T6SS is composed of approximately 15 conserved structural proteins and a variable number of accessory factors, which work in concert to secrete proteins extracellularly (Piepenbrink *et al*., 2016). Important components include Hcp, which forms a polymerized tubular structure that is secreted out of the cell and is essential for protein secretion, and VgrGs, which are present at the tip of this structure and can have effector activity or facilitate effector secretion (Ackerveken *et al*., 2016). The

T6SS bears striking similarity to bacteriophage, both structurally and functionally (Campanero-Rhodes *et al*., 2020). The presence of a T6SS was initially predicted bioinformatically for *A. baylyi*, and Hcp was subsequently detected in supernatants of *A. baumannii* ATCC 19606 (Jung *et al.*, 2011).

The genetic organization and sequences of T6SS genes are remarkably well conserved across *Acinetobacter* spp. Based on homology with T6SS genes in other bacteria, the single *Acinetobacter* T6SS locus includes most of the genes required for apparatus assembly and function (Turton *et al*., 2007). Notably, the main T6SS cluster does not contain *vgrG* genes, which are instead scattered throughout the genome. The VgrG proteins of *Acinetobacter*, which differ in number from strain to strain, do not seem to include effector domains (Thibau *et al*., 2020). Instead, bioinformatic analyses suggest that the proteins are most likely to mediate the secretion of adjacently encoded toxic effectors, with cognate immunity proteins being encoded nearby. However, no *bona fide* T6SS-dependent effectors have been experimentally characterized in *Acinetobacter*.

In *A. baylyi*, mutation of three PAAR proteins, which interact with VgrG proteins, results in loss of Hcp secretion, and one of those PAAR proteins has been experimentally determined to be secreted, although it is not clear whether the proteins have any effector functions themselves (M. L. Liou et al., 2014). The primary function of the T6SS in *Acinetobacter* seems to be to kill competing bacteria, and *Acinetobacter* spp. with active T6SSs are able to kill a wide variety of other bacteria, including other strains of the same species (Katiyi *et al*., 2020). T6SS expression often is tightly controlled and is activated only under certain conditions; the molecular mechanisms used to achieve this regulation are extremely diverse and complex and differ from organism to organism and even between strains of a given species (Li *et al*., 2019).

Although little is known about T6SS regulation in *Acinetobacter*, recent studies have provided insight into some of the regulatory mechanisms used by these organisms. T6SS activities vary widely in different strains and species of *Acinetobacter*, with some strains showing robust T6SSs and bacterial killing and others seeming to have inactive systems under laboratory conditions (Wang *et al*., 2017). However, the available data suggest that strains with T6SSs invariably express the main protein Hcp to at least some level, with variations in whether the protein is secreted (thus determining whether the system is active) (Nho, 2015). In *A. baumannii* ATCC 19606, Hcp is constitutively secreted in wild-type cells but is lost in mutants lacking lipid A, potentially due to membrane disruptions (Raetz & Whitfield, 2002). In *A. baumannii* ATCC 17978, T6SS activity is controlled by a plasmid (see below), and a chromosomally encoded histone-like nucleoid-structuring (H-NS) protein may also regulate the T6SS (Clark *et al*., 2016).

Several *A. baumannii* isolates harbor a resistance plasmid that encodes repressors of the T6SS (Metan *et al*., 2020). Upon spontaneous loss of this plasmid and subsequent loss of the repressors, the T6SS is activated, and the resistance genes are lost. The functional significance of this remains to be elucidated but, considering the tremendous amount of energy required for T6SS activity (Fernando & Kumar, 2013) and the fitness defects often caused by harboring multiple antibiotic resistance genes (Boo *et al*., 2006), this may represent a mechanism to maintain both systems while avoiding potentially deleterious effects of having them be active at the same time. In the absence of antibiotic pressure, *Acinetobacter* strains do not require resistance genes but are more likely to encounter competitors, thus losing the resistance plasmid and activating the T6SS, which could provide a competitive advantage. Because the cells that lose the plasmid will lose resistance to antibiotics,

this strategy could constitute an altruistic mechanism to ensure *Acinetobacter* population survival.

It should be noted that several recent multidrug resistant (MDR) *A. baumannii* strains seem to have permanently inactivated their T6SSs through chromosomal gene loss; it has been suggested that this may be a result of the antibiotic pressure being great enough to make it evolutionarily advantageous to completely lose the T6SS, rather than maintaining it in an inactive state (Dcosta *et al*., 2011). Although there are limited data, there appears to be a link between antibiotic resistance and T6SS status in *Acinetobacter*; strains that are multidrug resistant express but do not secrete Hcp, while those that are not multidrug resistant are more likely to have an active T6SS (Gales *et al.*, 2019b).

2.1.7 Outer membrane vesicles

A special case of protein secretion is the production of outer membrane vesicles (OMVs), which are blebs of outer membrane (OM) released from the bacterial cell surface (Kesavan *et al.*, 2020). There is significant debate regarding whether OMVs are produced by a directed process or simply represent cellular debris. Proteomic comparisons between the OM and OMVs of some bacteria have shown that the protein profiles differ between these two fractions, indicating that some OM proteins are excluded from OMV recruitment and suggesting that OMV formation is a directed process (Augustyniak *et al*., 2018). However, many studies also detected cytoplasmic proteins in OMV preparations, indicating that cell lysis could also be a major contributor to OMV formation (Nho *et al*., 2015). OMVs have been implicated in numerous biological functions, with particular attention being devoted to their role in virulence (Howard *et al*., 2018).

Several studies on OMVs in *Acinetobacter* have suggested that they have many functions, including roles in horizontal gene transfer, antibiotic resistance, and virulence. A wide variety of cargo types have been identified in OMVs from different *Acinetobacter* strains, including virulence proteins, antibiotic resistance determinants, and DNA (Gales *et al.*, 2019b; Metan *et al.*, 2020; Nicolet & Burnens, 2000; Schramm *et al.*, 2019). An important virulence factor of *A. baumannii*, OmpA, has also been found to be associated with OMVs, and OMVs have been suggested to act as a mechanism for delivery for this protein to host cells (Oh *et al.*, 2020). Furthermore, OmpA has been suggested to be involved in the biogenesis of OMVs (Nie *et al*., 2020). OMVs from *Acinetobacter* may have an important role in the development of novel therapeutics, as they can stimulate a strong immune response and are protective when administered as a vaccine (Aagaard *et al*., 2018; Chen, 2020; Ndungo *et al.*, 2018; Singh *et al.*, 2014).

2.2 Epidemiology of Acinetobacter calcoaceticus-A. baumannii (ACB) complex

Nosocomial infections (NIs) caused by *Acinetobacter* are among the main challenges of the health care system globally, particularly in the Hospital ICUs, accident and emergency units, and long-term care homes (Haque *et al*., 2018). Across the globe, about 1.7 million hospitalised patients yearly acquire NIs, and one (1) in every seventeen (17) patients is lost to NIs (Haque *et al*., 2018). A group of clinically important *Acinetobacter* (*A. baumannii, A. nosocomialis*, and *A. pittii*) called ACB complex is responsible for about 31% of all NIs cases worldwide *(*Duszynska *et al*., 2018; Sileem *et al.*, 2017). *Acinetobacter baumannii* is the most frequently encountered isolate among the ACB complex in the *Acinetobacter* nosocomial infection. Recent studies are increasingly reporting the involvement of *A. nosocomialis*

and *A. pittii* as the causative agents of *Acinetobacter* NIs (Chen *et al*., 2019; Chen *et al*., 2017; Wang *et al.*, 2013). Globally, the prevalence of NIs due to ACB complex varies between as low as 6.1% in the United States (Gupta *et al*., 2019; Logan *et al.*, 2019), Europe to 19.2% (Ayobami *et al*., 2020), in Asia, 60% (Rani *et al.*, 2017) and Africa 73.8% (Anane *et al.*, 2019).

In Indonesia, the report from Haji Adam Malik Hospital in Medan showed 17.44% of infections were caused by *A. baumannii* (Mayasari and Siregar, 2014). A study in the neonatal unit in the public hospital, Jakarta, showed 18.9% of *A. baumannii* positive culture from a total of 37 blood cultures from neonates with sepsis (Tjoa *et al.,* 2013). A similar study from one private hospital in North Jakarta; out of 92 identified *Acinetobacter* isolates from all kinds of specimens received in 2016, 14.1% were *A. baumannii* (Moehario *et al.*, 2020).

In Malaysia, a cross-sectional study on *Acinetobacter* infection in Hospital Universiti Sains Malaysia, Kelantan (HUSM), revealed that the prevalence of *Acinetobacter* NIs was 6.11% (58 *Acinetobacter* blood culture-positive cases from bacteraemia patients) (Deris *et al.*, 2009). A study from Hospital Universiti Kebangsaan, Malaysia, reported the prevalence of *Acinetobacter* species NIs at 19% and an overall mortality rate of 27.5% (Rozaidi *et al.*, 2001). However, some studies from various hospitals across Malaysia indicated that *Acinetobacter* species NIs are increasing. The Malaysian National Surveillance Antibiotic Resistance database reported that the prevalence of NIs due to carbapenem-resistant *Acinetobacter baumannii* presently stands nearly 60%, close to the levels reported in 2015 from Singapore (50%), Philippines (54.1%), and Thailand 73.7% (Rani *et al.*, 2017).

A study conducted in Germany to investigate the species distribution within the ACB complex involving 376 *Acinetobacter* isolates from hospitalized patients at 15 medical centers over five years. In this study, it was reported that *Acinetobacter nosocomialis* ranked the second most prevalent *Acinetobacter* species, 51.3% after *A. baumannii* 37.2% (Schleicher *et al.*, 2013). Also, in another study which compared the clinical features and antimicrobial susceptibilities of the clinically most important ACB complex involving 295 *Acinetobacter* isolates from patients with bloodstream infections (BSI) in 52 US hospitals in the United States of America showed that *A. baumannii* (63%) was the most prevalent species, followed by *A. nosocomialis* (21%), and *A. pittii* (8%). In a study by Wisplinghoff (2012), about one-third of *Acinetobacter* BSI was caused by *A. nosocomialis* or *A. pittii* (Wisplinghoff *et al.*, 2012). In another retrospective cohort study in Thailand on nosocomial infections due to ACB complex involving 222 patients reported *A. baumannii*, 197 (89%); *A. nosocomialis*, 18 (8%); and *A. pittii*, 7 (3%) (Chusri *et al*., 2014). The high prevalence of *Acinetobacter* NIs coupled with the high prevalence of mortality rate (27.5%) in Asia and 60% globally (Doughari *et al*., 2011) necessitate the search for new quick and easy diagnostic methods for *Acinetobacter* nosocomial in clinical laboratories.

2.3 Nosocomial infection due to *Acinetobacter* species

A nosocomial infection or also known as hospital-associated infections (HAIs) occurs within 48-72 hours to 30 days post-operation. *Acinetobacter* species is 10% among HAIs worldwide (Yang *et al.*, 2018). Prolonged stays in ICU requiring mechanical ventilation apart from trauma patients, thus are more susceptible to infections. In addition ventilator-associated pneumonia, infection of urinary tract, peritonitis, secondary meningitis, and bloodstream infections are also caused *by*

Acinetobacter species (Kohbodi & Noor, 2020). Urinary catheter-mediated bloodstream infections are caused by other species of *Acinetobacter*, with the clear tracing of contamination source. Reports of community spread due to *A. baumannii* are relatively rare; however, few scattered cases have been reported from time to time (Kinsella *et al*., 2015).

2.3.1 Respiratory tract infections

A. baumannii infections can pave the path for ventilator-associated pneumonia. Furthermore, differentiation between the actual infection site and spread to the upper respiratory tract is a grueling task. Of the total number of infections by *Acinetobacter* species, *A. baumannii* represents 26.7-47.9% of pneumonia (Wisplinghoff *et al*., 2000). Pneumonia caused by Gram-negative bacteria and *Acinetobacter* has similar diagnostic features and presents serious challenges to the subsequent treatment. Symptoms manifest as fever, leukocytosis, putrid sputum formation, and *de novo* infiltrates on radiograph or CT scan.

Sample collection involves both invasive and noninvasive methods. Noninvasive techniques are a collection of sputum, sputter exhaust cough, nasotracheal suctioning, and endotracheal aspiration. On the other hand, specimen obtained following invasive procedures includes bronchial brushings and bronchoalveolar lavage (BAL) A high mortality rate of 20 to 40% in *Acinetobacter* pneumonia was reported mainly due to the presence of co-occurring morbidity, finding the first line of antibiotic treatment and gravity of disease progression (Torres *et al*., 2018).

2.3.2 Bloodstream infections

A. baumannii causes bloodstream infections (BSIs), a major cause for concern, especially as a hospital-acquired infection. A large study conducted in the USA has demonstrated *A. baumannii* to be the most familiar organism related to nosocomial BSIs. It also demonstrates *A. baumannii* to be the 10th most common organism associated with nosocomial BSIs and accounts for about 1.3% of the overall monomicrobial nosocomial BSIs (Effatpanah *et al*., 2020). Pneumonia, trauma, surgery, dialysis, burns, and the existence of various catheters or intravenous lines (Charnot-Katsikas *et al*., 2018) are various liable indications towards BSIs. However, due to immunosuppression or respiratory failure at admission, there is triple the amount of risk towards the bacteremia). Pyrexia, leukocytosis, and repetitive blood cultures with the same positive genotypic isolate of *Acinetobacter* are the common clinical findings in bacteraemic episodes (Wisplinghoff *et al.*, 2012) *A. baumannii* associated with BSIs poses relative morbidity with an overall higher mortality rate (58%). The presence of comorbidities determines the prognosis of the patient (Hu and Robinson, 2010). Although, according to the latest investigation, *A. nosocomialis* and *A. pittii*- a low-virulent member of the ACB complex are the reasons for 30% of BSIs (Luo *et al.*, 2020). *Acinetobacter* bacteremia are commonly associated with mixed infections; although, it is not clear whether *A. baumannii* complex increases the pathogenicity when associated with mixed infections (Kettani *et al*., 2017). *Acinetobacter* isolates of about 10-15% have been collected from various blood cultures that belong to a variety of species which are not included in the ACB complex but are generally linked with other skin infection which are generally recommended to be examined with caution until other repetitive cultures are collected (Kettani *et al.*, 2017).