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Molecular Epidemiology and Insights into the Genomes of *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex

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Abstract

The genus *Acinetobacter* is amongst the most common causes of nosocomial bacterial infections. Even though *Acinetobacter baumannii* is the most frequent identified species causing a wide range of infections in humans, other species such as *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter ursingii* are sporadically observed as nosocomial pathogens. The four species that show similar phenotypes were grouped together as "*Acinetobacter calcoaceticus – A. baumannii* (ACB) complex", i. e. *A. baumannii*, *A. nosocomialis*, *A. pittii* and less clinically important *A. calcoaceticus*. Owing to the high complexity within *Acinetobacter* genus, it is difficult to differentiate *Acinetobacter* into species level and these organisms are frequently misidentified.

Acinetobacter spp. exhibit a great propensity to acquire antimicrobial resistance determinants and rapidly develop multidrug-resistant (MDR) phenotypes especially resistance to carbapenems, the last resort of antimicrobials to treat *Acinetobacter* infections. A high prevalence and endemic situations of carbapenem-resistant *A. baumannii* (CRAB) have been observed in multiple geographical areas such as Asia-Pacific and South America where predominant *A. baumannii* clonal lineages were noted. The international clone (IC) 2 is recognised as the most successful clone of *A. baumannii* causing outbreaks and persisting in hospital environments worldwide, particularly in Asia. Despite the increased amount of research on *Acinetobacter* epidemiology, potential virulence and evolution, little is known about the factors that may have contributed to the success of this well-known clone, IC2. The broad objectives of my PhD are to 1) develop a rapid method to assist in species identification of *Acinetobacter* non-*baumannii*, 2) determine the molecular epidemiology of *A. baumannii*, primarily from Thailand and 3) describe the genome of *A. baumannii* IC2 isolates from Thailand and compare these genome data with *A. baumannii* IC2 isolates from Japan, Malaysia and Singapore.

Firstly, a multiplex PCR was developed to detect intrinsic oxacillinases encoding genes (*bla*$_{\text{OXAs}}$), which assisted in rapid identification of multiple *Acinetobacter* species including *A. lwoffii*/*Acinetobacter schindleri*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus* and *Acinetobacter bereziniae*. Additionally, 30 novel *bla*$_{\text{OXAs}}$ variants were identified in this study. The investigation of *Acinetobacter* spp. collected worldwide revealed that acquired-type *bla*$_{\text{OXAs}}$ disseminated globally in *Acinetobacter* spp. as opposed to *A. baumannii* causing carbapenem resistance particularly in *A. pittii*. Carbapenem resistance was also observed in *A. pittii* from Australia and Thailand. Through the genomes of the two *A. pittii* strains ST119 and a novel ST655, several classes of antimicrobial resistance genes including a novel *bla*$_{\text{OXA-421}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-96}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{VEB-7}}$,

bla$_{\text{CRAB-2}}$, *floR, cmlA1, aar-2* and *dfrA10* were identified. This emphasises the importance of *A. pittii* as an impending multidrug resistance pathogen in this region.

Secondly, to explore an endemic situation of multidrug-resistant *Acinetobacter* spp., 300 non-repetitive ACB complex isolates, mainly *A. baumannii* from the largest tertiary hospital in Thailand were characterised for their molecular epidemiology and antimicrobial resistance mechanisms. Of these, 270 isolates were carbapenem-resistant and 92.2% resistant to amikacin. *A. baumannii* IC2 was the dominant clone of *A. baumannii* (80%) and *bla*$_{\text{OXA-23-like}}$ was detected in most of CRAB isolates. The genomes of 13 representative isolates of *A. baumannii* (*n*=11), *A. nosocomialis* (*n*=1) and *A. pittii* (*n*=1) were analysed via whole genome sequencing. Antimicrobial resistance island, AbaR4-type containing Tn*2006*, was found in all CRAB isolates. *armA* was the only 16S rRNA methylase gene found that caused resistance to amikacin (located within Tn*1548*) and in close proximity to macrolide resistance genes (*mphE* and *msrE*). *csu* locus, *bap*, *bfmRS* and *pga* locus associated with biofilm formation were found in all IC2 isolates in addition to the typical set of antimicrobial resistance genes; *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{ADC}}$, *strA*, *strB* and *tetB* in this clone. Therefore, the presence of arrays of antimicrobial resistance genes and biofilm-related loci may contribute to the spread and persistence of *A. baumannii* IC2 in this hospital.

Lastly, comparative genome analysis was performed on 21 representative *Acinetobacter* spp. isolates from Thailand and other countries, i.e. Japan, Malaysia and Singapore. The range of the CRAB genome size was 3.9 - 4.1 Mb with GC content approximately 39%. Interestingly, the susceptible *A. baumannii* genome size was 3.7-3.8 Mb. The size difference was due to the absence of resistance genes and regions for bacterial competition and biofilm formation in susceptible isolates. The genetic contexts of key antimicrobial resistance genes and resistance islands were investigated. Our study confirmed that the antimicrobial resistance genes and other genomic features of IC2 isolates were homogenous. The diversity was mainly found in the composition of the antimicrobial resistance genes of IC2 isolates and locus for capsule synthesis (K locus). The genome and composition of antimicrobial resistance genes between IC2 isolates from Thailand and all IC2 from other countries were closely related. Two IC2 carbapenem-susceptible isolates from Japan harboured less antimicrobial resistance genes (*bla*$_{\text{OXA-66}}$, *strA, strB, sul2* and *tetB*). The locus for the outer core of lipid A-core moiety (OC locus) was generally conserved - OCL1 was a common type within IC2. Such variations observed of the K locus may impact in the difficulties to generate human immune response to *A. baumannii*. Lastly, one key feature of IC2 was the integrity of all chromosomal regions for type VI secretion system (T6SS) and biofilm formation (*csu*). In contrast, these regions in non-

IC2 isolates were disrupted. This may indicate the incapability of non-IC2 isolates to persist in the hospital environment.

The study of various aspects of carbapenem-resistant *Acinetobacter* spp. in our region has revealed specific insights into this pathogen locally and globally. These include the species identification, molecular epidemiology and the genome of *Acinetobacter* spp. Genomic analysis has described all necessary attributes of the superiority of IC2 to spread further under antimicrobial pressure and harsh environments. Further research is required to understand greater detail of these many unique findings.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed publications

1. **Kamolvit W**, Sidjabat HE, Paterson DL. Molecular epidemiology and mechanisms of carbapenem resistance of *Acinetobacter baumannii* in Asia and Oceania. Microb Drug Resist. 2015. Feb 25. [Epub ahead of print]. *Impact factor: 2.524.*

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3. **Kamolvit W**, Higgins PG, Paterson DL, Seifert H. Multiplex PCR to detect the genes encoding naturally occurring oxacillinases in *Acinetobacter* spp. J Antimicrob Chemother. 2014 Apr;69(4):959-63. PubMed PMID: 24288029. *Impact factor: 5.439.*

4. Zander E, Fernández-González A, Schleicher X, Dammhayn C, **Kamolvit W**, Seifert H, Higgins PG. Worldwide dissemination of acquired carbapenem-hydrolysing class D β-lactamases in *Acinetobacter* spp. other than *Acinetobacter baumannii*. Int J Antimicrob Agents. 2014 Apr;43(4):375-7. PubMed PMID: 24612983. *Impact factor: 4.259.*

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Chapter 1.

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In addition to the contributions outlined above, Dr Anna Sartor and Nattaya Tamrongsakulsiri, B.Sc. assisted with laboratory work including genotypic characterisation presented in chapters 3.

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Keywords

Acinetobacter baumannii, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, oxacillinases, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-51-like}}$, *armA*, international clone, genome, biofilm.

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Table of Contents

List of abbreviations used in the thesis

Abbreviation	Term
A. baumannii	*Acinetobacter baumannii*
A. bereziniae	*Acinetobacter bereziniae*
A. calcoaceticus	*Acinetobacter calcoaceticus*
A. johnsonii	*Acinetobacter johnsonii*
A. junii	*Acinetobacter junii*
A. lwoffii	*Acinetobacter lwoffii*
A. nosocomialis	*Acinetobacter nosocomialis*
A. pittii	*Acinetobacter pittii*
A. radioresistens	*Acinetobacter radioresistens*
A. schindleri	*Acinetobacter schindleri*
A. ursingii	*Acinetobacter ursingii*
AbaR	*Acinetobacter baumannii* Resistance island
ACB	*Acinetobacter calcoaceticus – Acinetobacter baumannii* Complex
AFLP	Amplified Fragment Length Polymorphism
APAC	Asia-Pacific
BAL	Broncheoalveolar lavage
Bap	Biofilm-Associated Protein
bfmRS	Biofilm Formation Regulatory System
CHDL	Carbapenemase hydrolysing class D beta-lactamase
CSF	Cerebrospinal fluid
CC	Clonal Complex
CLSI	Clinical and Laboratory Standards Institute
CRAB	Carbapenem-resistant *Acinetobacter baumannii*

CRSB	Carbapenem-susceptible *Acinetobacter baumannii*
csu	Chaperone-usher pili assembly system
DST	Disk Susceptibility Testing
DVL	DiversiLab – rep-PCR pattern
ESBL	Extended Spectrum Beta Lactamase
GIM	German Imipenemase
IC	International Clone
ICU	Intensive Care Unit
IMP	Imipenemase
IS	Insertion Sequence element
KL	Capsule Locus
LBA	Luria Bertani Agar
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentration
MLST	Multi Locus Sequence Typing
NDM	New Delhi Metallo-β-lactamases
OCL	Outer Core Locus
OPD	Out Patient Department
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
SIM	Seoul Imipenemase
SG	Sequence Group

SNP	Single Nucleotide Polymorphism
ST	Sequence Type
T6SS	Type VI secretion system
Tn	Transposon
VIM	Verona integron-encoded metallo-β-lactamase or Verona imipenemase
WGS	Whole Genome Sequencing
WW Lineages	Worldwide Lineages

Chapter 1. Introduction

1.1 Synopsis

Nosocomial infections represent an important problem in public health and are increasing throughout the world. There has been an increase in morbidity and mortality due to such infections [1]. The difficulty to treat and eradicate microorganisms causing nosocomial infections from the hospital environment is a major challenge to physicians and healthcare workers [2].

Gram negative pathogens have been the focus of recent clinical attention due to their increasing frequency in causing hospital-acquired infections. In this group, *Acinetobacter* spp. is emerging as a pathogen that frequently causes infections in patients in intensive care units [3]. The most common *Acinetobacter* spp. involved in hospital infections is *Acinetobacter baumannii* [4]. Within the last three decades, *A. baumannii* has exhibited a high propensity to develop antimicrobial resistance, including resistance to carbapenems, one of the last line drugs for treatment *A. baumannii* infection, which leaves us with few remaining treatment options (i.e. tigecycline and colistin).

A high prevalence of carbapenem-resistant *Acinetobacter* spp. was observed throughout the world, particularly in the Asia-Pacific region [5, 6]. In multiple locations throughout the world pandrug-resistant strains have been identified [7-10], implying resistance to all commercially available antimicrobials. The acquisition of plasmids, transposons or integrons, carrying clusters of genes harbouring resistance to several antimicrobial families simultaneously, plays an important role in acquiring multidrug resistance.

The molecular epidemiology of multidrug-resistant *A. baumannii* from different countries has been studied with increasing intensity in recent years. The vast majority of isolates disseminated worldwide belong to just one or two clones, which were described as International clone (IC) 1 and 2 [11]. The global spread of successful international clones underlines the importance of molecular epidemiologic and genome-wide studies in order to obtain a greater understanding of population genetics and adaptive mechanisms amongst these powerful these clones.

In the paper “Molecular epidemiology and mechanisms of carbapenem resistance of *Acinetobacter* spp. in Asia and Oceania”, I focused on the studies from Asia and Oceania to illustrate and better understand the population structure of carbapenem-resistant *Acinetobacter* spp. in these regions. Several typing methods, epidemiology, and mechanisms of carbapenem resistance in *A. baumannii*

were outlined. In addition, we elucidated the distribution of oxacillinases and metallo-β-lactamases, the most important β-lactamase classes causing carbapenem resistance in *A. baumannii*.

1.2 Molecular epidemiology and mechanisms of carbapenem resistance *Acinetobacter* spp. in Asia and Oceania

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Molecular Epidemiology and Mechanisms of Carbapenem Resistance of *Acinetobacter* spp. in Asia and Oceania

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Acinetobacter baumannii is emerging as a pathogen that is commonly involved in nosocomial infections. *A. baumannii* has exhibited the ability to develop multidrug resistance (MDR), including resistance to carbapenems, the last-line class of antibiotics to treat these infections. In particular, MDR *A. baumannii* International Clone (IC) 2 has disseminated worldwide causing substantial problems in hospitals, including in Asia and Oceania. The global spread of this clonal lineage emphasizes the importance of tracking molecular epidemiology to obtain greater understanding of the population dynamics of *A. baumannii*. Carbapenem resistance in *A. baumannii* occurs mainly as a result of acquisition of OXA-type carbapenemase genes, and to some extent by acquisition of metallo-β-lactamase genes. The acquisition of carbapenemase genes, particularly the $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$, and $bla_{\text{OXA-58}}$, by specific clonal lineages may be one of the attributes responsible for the relative homogeneity of the MDR *A. baumannii* population.

Introduction

Acinetobacter is a nonlactose fermenting and strictly aerobic gram-negative genus.[22] This genus currently comprises 44 species; 9 genomic species and 35 validly named species (www.bacterio.cict.fr/a/acinetobacter.html). Owing to their high phenotypic and genetic similarities, four of these species are grouped as the ''*A. calcoaceticus-A. baumannii* (ACB) complex.'' They include *Acinetobacter calcoaceticus* (genomic species 1), *Acinetobacter baumannii* (genomic species 2), *Acinetobacter pittii* (genomic species 3), and *Acinetobacter nosocomialis* (genomic species 13TU). Among the ACB complex, *A. baumannii*, *A. pittii*, and *A. nosocomialis*, which are also recognized as the *A. baumannii* group, are the most clinically important species. Meanwhile, *A. calcoaceticus* is considered as a nonpathogenic environmental organism and is rarely isolated from clinical specimens.[45,71]

Due to high similarities of phenotypic and genotypic characteristics, it is difficult to identify *Acinetobacter* spp. into their species level.[6] Available automated systems used in routine diagnostic laboratories, such as API-20NE, Vitek 2®, and Phoenix, are not able to distinguish the species among the ACB complex. Furthermore, phenotypic and genotypic methods, such as DNA-DNA hybridization and amplified rRNA gene restriction analysis are time-consuming.[7,99] Sequencing methods of *rpoB* and 16S–23S rRNA gene spacer region are reliable for such species identification.[11,55] A rapid PCR-based method for *gyrB* is another reliable tool for species differentiation among the ACB complex.[33] In this review, we verified that publications within the literature had applied proper methods for species identification. The term ''*Acinetobacter* spp.'' in this review was used where the methods could not identify species level of *Acinetobacter* spp.

Within the last few decades, *A. baumannii* has alarmingly emerged as one of the most important nosocomial pathogens. Infections caused by *A. baumannii* as a nosocomial pathogen includes ventilator-associated pneumonia, bloodstream infection, wound infection, and meningitis. There are few therapeutic options that can overcome this organism.[77] Carbapenems are one such remaining option as the last-line drugs for treatment of infections caused by *A. baumannii*. Unfortunately, resistance to carbapenems has become common among *A. baumannii* worldwide. It is worth noting that *A. baumannii* is naturally resistant to several antibiotics such as ampicillin, narrow-spectrum cephalosporins, trimethoprim, and ertapenem.[56] A high prevalence of carbapenem-resistant *A. baumannii* is observed in several geographic areas such as Asia-Pacific (APAC), Latin America, and the Mediterranean.[15,35,44,96] The SENTRY antimicrobial surveillance program has shown a continuing decrease in the imipenem susceptibility rates among *Acinetobacter* spp. collected from different regions (Fig. 1).

The susceptibility rate to imipenem in the APAC region declined from 73.7% in 2001–2004 to 37.4% in 2009

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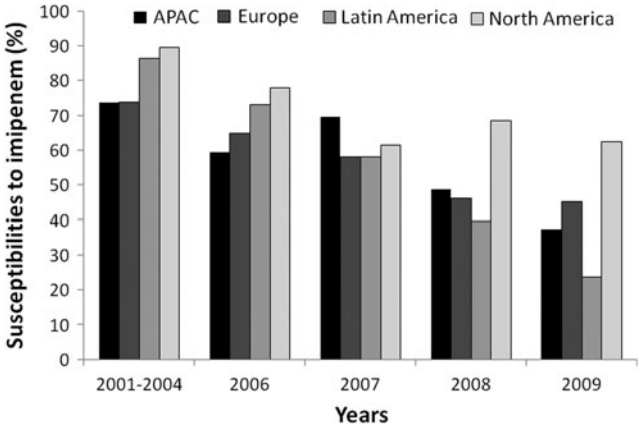

FIG. 1. Imipenem susceptibility rates in *Acinetobacter* spp. from Asia-Pacific (APAC), Europe, Latin America, and North America (the SENTRY surveillance program).[23,24]

(Fig. 1).[26,27] This high rate of carbapenem resistance was observed in most countries from this region. Moreover, pandrug-resistant *A. baumannii* isolates (resistant to all available class of antibiotics) have also been increasingly identified in Asia.[4,16] This highlights the increasing trend of antibiotic resistance in *A. baumannii* in the APAC region, especially resistance to carbapenems.

Molecular Typing Methods for *A. baumannii*

A number of molecular typing methods have been used to understand the molecular epidemiology of *A. baumannii*. The most commonly used typing techniques include (1) DNA-based fingerprinting methods, pulsed-field gel electrophoresis (PFGE),[85] amplified fragment length polymorphism analysis,[21] and multiple-locus variable-number tandem-repeat analysis[96] and (2) PCR-based and sequencing methods, repetitive sequence-based PCR (rep-PCR) analysis,[32] multilocus sequence typing (MLST),[5,20] and sequence-based typing and their allele-specific multiplex PCRs.[95] Whole-genome sequencing has also been used for this purpose.[1,87]

PFGE or restriction analysis of chromosomal bacterial DNA is still currently considered as the gold standard for epidemiologic typing and used in numerous *A. baumannii* outbreak studies showing high discriminatory power.[85] However, PFGE is laborious, and thus, it is not a suitable technique for rapidly resolving the molecular epidemiology of outbreaks and is not suitable for comparing typing results between laboratories. Amplified fragment length polymorphism (AFLP) analysis has been used as a reference method to identify outbreak clones in Europe, initially named European clones I, II, and III, which had later spread worldwide[21,98] and are now known as worldwide clone, global clone, or international clone (IC) 1, 2, and 3.[22,32] Up to now, there is no consensus regarding the terminology. In recent literature, *A. baumannii* outbreak strains have been designated as international clones over others, such as worldwide clone or European clone.[20,113] Therefore, ''international clone'' (IC) will be used in this review.

MLST is an objective method of typing microorganisms that are suitable for population-based studies and global epidemiologic analysis as it allows one to place tested isolates among global isolates registered in centralized *A. baumannii* MLST databases.[5,20] This method is based on comparison of the sequences of internal fragments of seven housekeeping genes.[5] Two MLST schemes are available for *Acinetobacter*. The first MLST scheme was established by Bartual *et al.* and is maintained at PubMLST (http://pubmlst.org/abaumannii/). This scheme uses *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* genes.[5,106] The second MLST scheme is maintained at the Pasteur Institute's MLST database (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html). The method uses *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* genes, sharing three of them with the PubMLST scheme.[20]

In Asia and Australia, PubMLST is used more frequently, while groups in Europe recently prefer to use the Pasteur Institute's scheme.[19,28,39] Both MLST schemes were applicable to non-*baumannii Acinetobacter* spp.[104,106] However, the Pasteur MLST database has more designated STs for non-baumannii species and strains than PubMLST. This suggests that Pasteur MLST may be suitable for global epidemiology study of non-*baumannii Acinetobacter* spp. We have performed a population study on multidrug resistance (MDR) *A. baumannii* on a global scale using the PubMLST scheme. Our results showed that clonal complex 92 (CC92) was the largest and most geographically diverse CC, which corresponded to IC 2 based on previous typing methods, that is, AFLP and rep-PCR.[83]

Multiplex PCR is an alternative method to differentiate clonal lineages of *A. baumannii*. This method is a sequence-based typing method for three specific genes: *ompA*, *csuE*, and $bla_{\text{OXA-51-like}}$ which was developed by Turton *et al.*[95] Owing to higher numbers of polymorphism found in these three genes, this approach has the possibility for greater discrimination than schemes based on housekeeping genes such as MLST.[94,95] Through this method, 96 carbapenem-resistant *A. baumannii* isolates from hospitals in 17 European countries were characterized. Seven different sequence groups (SGs) were identified, and three of these (SG 1, 2, and 3) corresponded to IC 2, 1, and 3, respectively. The majority of isolates belonged to IC 2 and 1, and the remainder belonged to four novel groups.[94] A group in Australia also used this method to characterize Australian isolates, where the majority of isolates belonged to IC 2 and 1.[28]

rep-PCR is another useful typing method, one of which is a semiautomated and commercially available method called DiversiLab™ (BioMérieux). This method utilizes a microfluidic LabChip on a bioanalyzer to analyze the amplicons and uses built-in software to build the dendrograms. This method offers the advantages of rapid turnaround time, ease of use, and the ability to maintain libraries of all typed isolates that can be valuable for a future epidemiologic study. Higgins *et al.* investigated a large global cohort of imipenem-nonsusceptible *A. baumannii* isolates from 32 countries using the DiversiLab system.[32] The result showed the presence of at least eight distinct clonal clusters, which were assigned as worldwide (WW) lineages 1–8. Three of these clusters represented IC 1, 2, and 3 (WW 1, 2, and 3, respectively) with a predominance of IC 2. Higgins *et al.* also showed results comparable to those generated by the DiversiLab system and those from PFGE and MLST.[83,84] This suggests that the DiversiLab system is one of the more reliable techniques for the global epidemiologic study of *A. baumannii*.

Molecular Epidemiology of *A. baumannii* in Asia and Oceania

The molecular epidemiology of *A. baumannii* in Asia and Oceania has been studied with increasing intensity in recent years using various typing methods. MLST has been most frequently used. To better understand the molecular epidemiology of *A. baumannii* in Asia and Oceania, here, we have generated a snapshot of the *A. baumannii* population structure from Asia and Oceania available in the PubMLST database. This snapshot was generated using eBURSTv3 analysis (http://eburst.mlst.net/v3/enter_data/single/) (Fig. 2). We included *A. baumannii* isolates from nine countries: Australia ($n=51$), China ($n=125$), India ($n=22$), Japan ($n=33$), South Korea ($n=71$), Malaysia ($n=2$), Singapore ($n=4$), Thailand ($n=12$), and Vietnam ($n=11$).

A total of 331 *A. baumannii* isolates from 170 different STs were available in the PubMLST database from Asia and Oceania. These were grouped in 17 CCs and 68 singletons (Fig. 2). The most predominant CC was CC92 ($n=161$, 48.6%), which included isolates from all countries except India. CC92 corresponds to IC 2. Among CC92, ST92 ($n=72$), ST75 ($n=23$), and ST138 ($n=10$) were the most identified STs in the PubMLST database. ST92 was recovered from Australia, China, Japan, South Korea, and Thailand from 2000 to 2010.

Additionally, we have sought additional ST information from publications from the region available in PubMed. ST92 was also found in India, the Philippines, and Taiwan, however, these data were not available through the PubMLST database.[50] ST75 (also part of CC92) was reported as a major ST that caused outbreaks in hospitals in China and South Korea.[17,60,74,114] A study from South Korea demonstrated that ST75 and ST138 have increased in 2008–2010.[74] The isolates of these two STs showed higher carbapenem resistance rates than isolates of ST92, which was the most prevalent ST in 2003–2007.

In Asia and Oceania, IC 1 and IC 3 are relatively uncommon compared to IC 2. CC109 ($n=15$), which corresponds to IC 1, was the second most common CC in Asia and Oceania. More than 50% of the isolates clustered in this CC were recovered from Australia. ST109 was the predicted founder, which was first reported from Australia in 1997. This ST was also found in Japan and South Korea.[89] CC110, which corresponded to IC 3, consisted of eight isolates from South Korea (three), India (two), Australia, Japan, and Vietnam. CC561, a newly described CC has been found in bloodstream isolates in South Korea (2010–2011) and not associated with IC 1, IC 2, or IC 3. Furthermore, *A. baumannii* can be isolated from feces of pigs and cattle. However, none of these strains was associated with IC 1, IC 2, or IC 3, which are the common clones of *A. baumannii* strains in humans.[30] This shows that lineages of *A. baumannii* from animals and humans are distinct.

In conclusion, a few major clonal lineages, including IC 1, 2, and 3, were responsible for the spread of *A. baumannii* in Asia and Oceania. CC92 (corresponding to IC 2) was the most prevalent clonal lineage recovered from this region (48.6%). There was an emergence of a single CC, CC561, which was found only in South Korea (Fig. 2). Even though CC92 is the most commonly identified clone over a decade, a shift of STs into more carbapenem-resistant ones was observed within CC92. This suggests that carbapenem resistance may be responsible for an evolution within successful clonal lineages, which may lead to a decrease in the heterogeneity of the *A. baumannii* population.

Mechanisms of Carbapenem Resistance in *A. baumannii*

Several molecular mechanisms are responsible for conferring carbapenem resistance in *A. baumannii*. The most common mechanism of carbapenem resistance is the production of carbapenem-hydrolyzing β-lactamases; oxacillinases (OXA-type carbapenemases) and metallo-β-lactamases (MBLs). Alteration of penicillin-binding proteins and loss of outer membrane proteins,[18,42,69,100] efflux pump mechanisms, and other β-lactamases are also commonly implicated in carbapenem resistance of *A. baumannii*.[100]

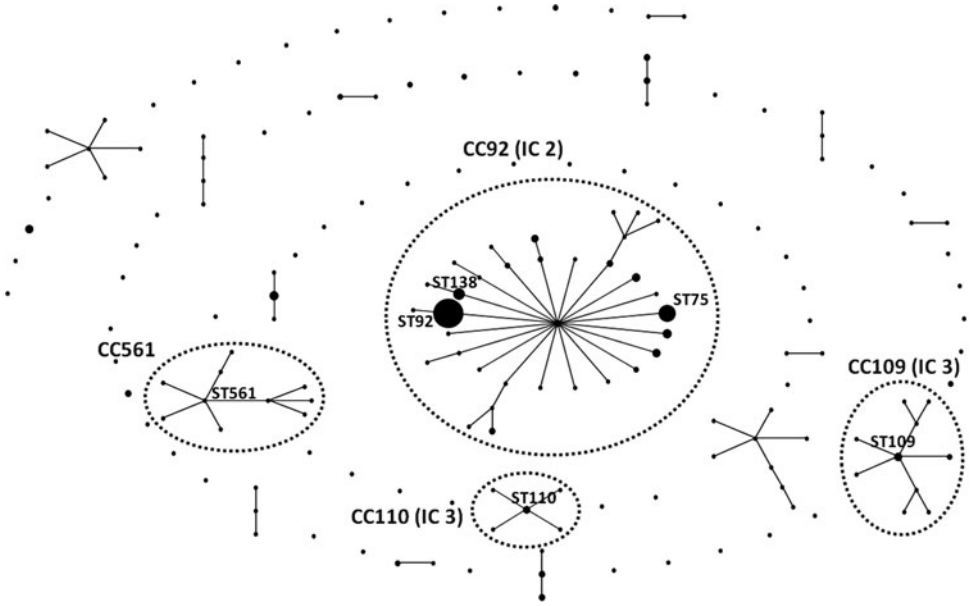

FIG. 2. A population snapshot of *Acinetobacter baumannii* isolates form Asia and Oceania (data from PubMLST). The number of epidemic and clonal complexes (CCs) with corresponded international clones (ICs) is indicated (*dash line*). The *circle* represents an ST. The relative size of the *circles* indicates their prevalence in the PubMLST database. The *lines* connecting each *circle* represent single locus variant STs that differ in only one of the seven housekeeping genes.

Oxacillinases

The name oxacillinase refers to the ability to hydrolyze the isoxazolyl penicillin, oxacillin, much better than the classical penicillins.[10] The group of OXA-type carbapenemases exhibits carbapenem-hydrolysing activities, in contrast to most OXA-type β-lactamases which do not hydrolyse carbapenems. Most of these enzymes are weak carbapenemases. However, when overexpressed they are able to confer carbapenem resistance.[24]

To date, several variants of acquired type OXAs found in *A. baumannii* have been identified (Fig. 3A). Four major subgroups of OXA-type carbapenemases include both (1) acquired types (OXA-23, OXA-40, and OXA-58 like), where their genes have been found either in the chromosome or plasmid of some but not all *A. baumannii* isolates and (2) the naturally occurring chromosomal OXA-51-like carbapenemase.[97] Additional groups of acquired OXA-type carbapenemases such as OXA-143 and OXA-235 like have also recently been identified in *A. baumannii*.[34,35]

The acquisition mechanisms for OXA-encoding genes in *A. baumannii* are not thoroughly understood. The $bla_{\text{OXA-23-like}}$ and the $bla_{\text{OXA-40-like}}$ genes have been reported to be encoded on both the chromosome and plasmids.[24,68,77,102] The $bla_{\text{OXA-58-like}}$ genes were frequently identified as plasmid mediated. Multiple genomic studies have revealed the pivotal role of insertion sequence (IS) elements. The presence of IS*Aba1* upstream of $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-235-like}}$ as well as intrinsic chromosomal $bla_{\text{OXA-51-like}}$ provides promoter sequences leading to overexpression of these downstream genes conferring resistance to carbapenems.[34,81,97] IS*Aba1*, IS*Aba2*, IS*Aba3*, and IS*8* have been shown to enhance the expression of $bla_{\text{OXA-58}}$.[82] The overexpression of $bla_{\text{OXA-40-like}}$ and $bla_{\text{OXA-143-like}}$ is not associated with IS elements and their native promoters may be sufficient for overexpression.[92,111]

Metallo-β-lactamases

MBLs, Ambler class B enzymes, bear two zinc ions in their active sites and are capable of hydrolyzing all β-lactam antibiotics except aztreonam. IMP, VIM, SIM, and NDM, a recently described MBL, have been identified in *Acinetobacter* spp. from Asia and Oceania.[76,81,101] Among these MBLs, IMP was the most commonly reported in *A. baumannii* until 2007. However, NDM has often been reported in various *Acinetobacter* species since its emergence in 2009.[101,110] Generally, MBLs are less commonly found in *A. baumannii* than the OXA-type carbapenemases, most of which, with the exception of NDM, are captured by integrons as gene cassettes. NDM in comparison is found to mobilize through a composite transposon, Tn*125*, among *Acinetobacter* spp.[79] Furthermore, *A. baumannii* has also been theorized to be the platform for the origin of NDM.[93]

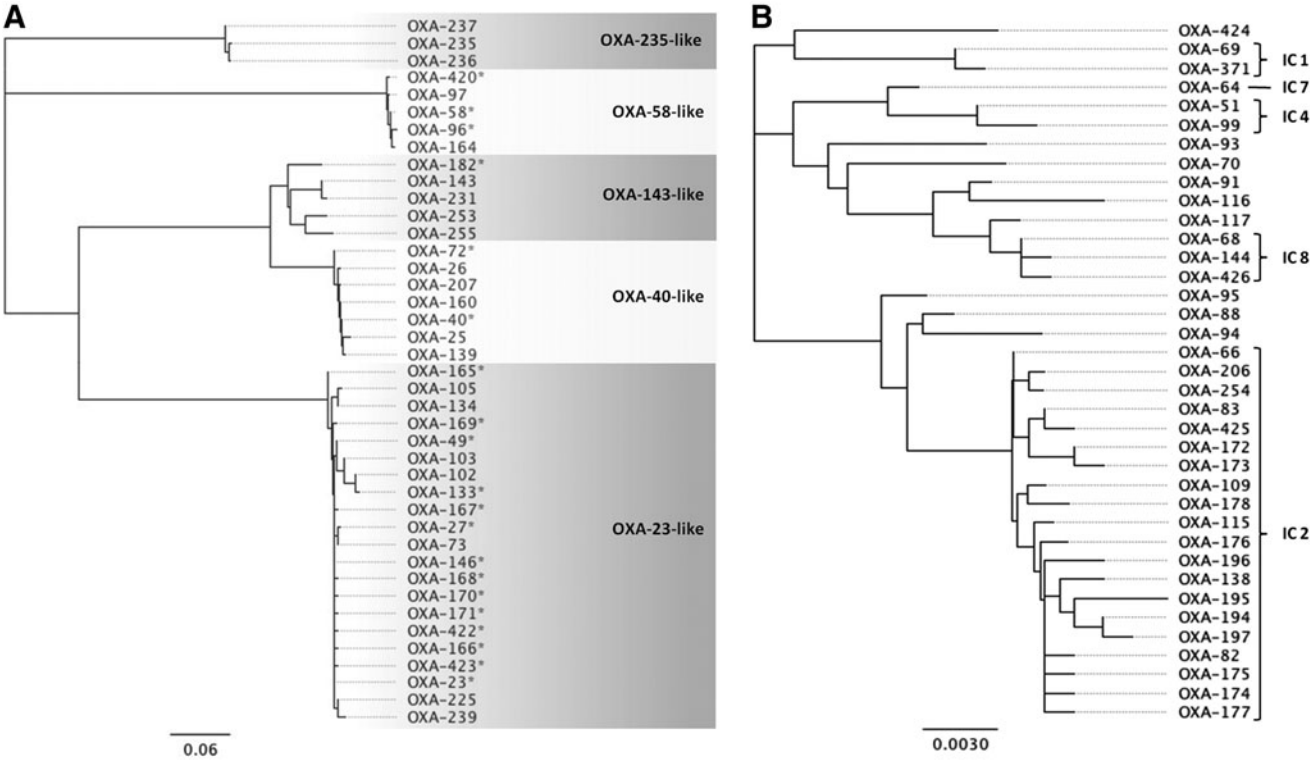

FIG. 3. **(A)** Unrooted neighbor-joining tree based on amino acid of five groups of OXAs (OXA-23 like, OXA-40 like, OXA-58 like, OXA-143 like, and OXA-235 like) that were identified in *Acinetobacter* species. *Horizontal bar*; 6% sequence divergence. *OXA enzymes were reported from Asia and Oceania. **(B)** Unrooted neighbor-joining tree based on nucleotide sequences of all $bla_{\text{OXA-51-like}}$ identified in *A. baumannii* from Asia and Oceania, except $bla_{\text{OXA-138}}$, $bla_{\text{OXA-194}}$ to $bla_{\text{OXA-197}}$ that were found in *Acinetobacter nosocomialis*. *Horizontal bar*; 0.3% sequence divergence. ICs associated with each cluster of $bla_{\text{OXA-51}}$ variants were indicated.[105] Both trees were generated by using Geneious version 7.1 created by Biomatters (www.geneious.com).

A rarely reported MBL, the GIM-1 enzyme, has been recently reported in *A. pittii* isolates in Germany, but has not yet been recovered in Asia and Oceania.[44]

Distribution of OXAs and MBLs in Asia and Oceania

The distribution of acquired type OXA and MBL genes is very diverse (Table 1). The acquisition of the $bla_{\text{OXA-23-like}}$ has become the most common cause of carbapenem resistance in *A. baumannii* in Asia and Oceania.[50] OXA-23 was initially described in 1985 as ARI-1[75] and is the most widely spread acquired OXA carbapenemase in this region and worldwide. Other variants of OXA-23, such as OXA-27 (Singapore), OXA-49, OXA-146, OXA-422, and OXA-423 (China), were identified in carbapenem-resistant *A. baumannii* isolates (Table 1).[2,103] OXA-165 to OXA-171 were reported from Thailand (accession no. HM488986 to HM488992). OXA-23-like enzymes have also been found in *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus*.[51,52] Several variants of OXA-23 like (OXA-23, OXA-102, OXA-103, OXA-105, OXA-133, and OXA-134) were found on the chromosome of *Acinetobacter radioresistens* suggesting that *A. radioresistens* is the progenitor of the $bla_{\text{OXA-23-like}}$ genes.[66,80]

The prevalence of the OXA-40-like cluster causing carbapenem resistance in *Acinetobacter* spp. from Asia and Oceania is low. OXA-72 was first identified in *A. baumannii* from Thailand in 2004 (GenBank accession no. AY739646). This enzyme was recovered from *A. baumannii* isolates from India and southern Taiwan, where OXA-72-producing *A. baumannii* has been reported to cause hospital outbreaks.[54,63,65] OXA-40-like carbapenemases have been found in non-*baumannii* species such as *A. pittii* and *A. nosocomialis*.[15,103]

OXA-58-like enzymes comprise OXA-58, OXA-96, OXA-97, OXA-164, and OXA-420. OXA-58 in *A. baumannii* was detected across Asia and Oceania, including Australia, China, India, Singapore, South Korea, and Taiwan (Table 1). Interestingly, the ratio of OXA-58-like enzymes among carbapenem-resistant *Acinetobacter* was above 40% from certain hospitals in Taiwan.[40,61] OXA-96, which differs from OXA-58 by one amino acid substitution, was only described in an *A. baumannii* isolate from Singapore in 2006.[51] OXA-420 has been recently detected in *A. baumannii* from Nepal (GenBank accession no. AB983359). Of note, OXA-58-like enzymes were frequently identified in *A. pittii* isolates from Singapore and Taiwan.[40,51,52]

OXA-182, a member of OXA-143-like enzymes, has emerged in South Korea.[49] This enzyme shares 93% identity with OXA-143, which is reported to be highly prevalent in Brazil.[35,67] The study from South Korea showed that OXA-182 was identified in imipenem-nonsusceptible *A. baumannii* isolates recovered as early as 2002 and in *A. nosocomialis* from year 2004 to 2007.[49]

OXA-51-like enzymes are intrinsic for *A. baumannii* and have also been recovered in Taiwan from *A. nosocomialis*.[9,62] OXA-51 like is the most diverse group of oxacillinase with more than 100 variants identified.[24,78] Furthermore, the sequences of OXA-51-like genes showed a correlation with their epidemiologic grouping, that is, OXA-69, OXA-66, OXA-71, OXA-51, OXA-65, OXA-64, and OXA-68 correspond to IC 1, 2, 3, 4, 5, 7, and 8, respectively.[25,112] OXA-51-like enzymes that were identified in Asia and Oceania are shown in Fig. 3B. OXA-66 was found in Australia, China, Hong Kong, India, Japan, Singapore, South Korea, and Taiwan.[23,25,51,64,72,103] OXA-138 and OXA-194 to OXA-197 recovered from *A. nosocomialis* in Taiwan were also clustered with OXA-66. OXA-69 identified in Australia, India, and Pakistan was grouped with OXA-371 from Nepal.[25] OXA-51 was detected in Japan and India.[23] OXA-68 (Hong Kong and Singapore)[8] was clustered with OXA-144 (Pakistan) and OXA-426 (China). OXA-64 was only reported from Singapore.[29] There was no report of OXA-65 and OXA-71 in Asia and Oceania.

Even though MBLs are less frequently identified in *A. baumannii* in Asia and Oceania, a relatively high prevalence of MBL-producing *Acinetobacter* spp. isolates was observed in some countries, such as India and South Korea.[58,73,86] Several variants of IMP-type enzymes were identified in Japan, including IMP-1, IMP-11, and IMP-19 (Table 1). IMP-2 was identified in *A. baumannii* isolates from a hospital in India.[73] This enzyme shares 84.9% amino acid identity with IMP-1. IMP-4, which had 95.6% amino acid identity with IMP-1, was first described in *Acinetobacter* spp. isolates from Hong Kong[14] and was subsequently reported from Australia and Singapore as well.[51,76] IMP-8 and IMP-14 were only identified in Taiwan and Thailand, respectively.[46,59]

VIM-like enzymes were sporadically reported from India, South Korea, and Taiwan.[49,59,73] SIM-1 was identified in China and South Korea.[58,115] NDM-1 was recently recovered from *Acinetobacter* spp. isolates from India, Bangladesh, Pakistan, Japan, and China.[101] These isolates from China included nonclinical samples recovered from hospital sewage and meat-producing animals.[105,117]

MBLs were identified in several *Acinetobacter* species, including *A. baumannii, A. baylyi*, *A. bereziniae*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *and A. junii*, *A. lwoffii*, *A. nosocomialis*, *and A. pittii*.[53,59,76,88,101,107,108,115] MBLs identified in *Acinetobacter* spp. are listed in Table 1.

Conclusion

Carbapenem resistance in *A. baumannii* has had a drastic increase in Asia and Oceania within the last decade. The increase of carbapenem resistance in non-baumannii *Acinetobacter* spp. has also been observed. Production of OXA carbapenemases and MBLs is the main mechanism of carbapenem resistance in *A. baumannii*. OXA-23-like enzymes are the most commonly identified carbapenemases in *A. baumannii* from Asia and Oceania. Other groups of OXA carbapenemases and MBLs are sporadically reported from different countries. It is worth noting that NDM can also be isolated from meat-producing animals in China. Thus, the spread of NDM-producing *Acinetobacter* may be hastened by its appearance in the food chain and subsequently become a real threat in Asia and Oceania.

CC92, which corresponds to IC 2, is the most successful and widely disseminated clone of *A. baumannii* in Asia and Oceania. Carbapenem resistance may be one of the adaptive mechanisms responsible for a decrease in heterogeneity among the *A. baumannii* population. To address this increase of carbapenem resistance *Acinetobacter* spp., proper species identification and typing methods are essential for early detection and monitoring in epidemiology investigations. Factors that have contributed to the ability of *Acinetobacter* spp. to spread and persist in the hospital

Table 1. Acquired-Type OXAs and MBLs in *Acinetobacter* Identified in Asia and Oceania

	Enzymes	*Species*	*Country*	*Reference/accession no.*
OXA-type carbapenemases	OXA-23 like			
	OXA-23	*Acinetobacter baumannii*	AU, CH, JP, HK, IN, SP, KO, TW, TH, VT	13,36,37,48,52,53,60,83,90,91
		Acinetobacter nosocomialis	CH, KO, SP	50,52,103
		Acinetobacter pittii	CH, KO, SP	51,60,103
	OXA-27	*A. baumannii*	SP	2
	OXA-49	*A. baumannii*	CH	AY288523
	OXA-146	*A. baumannii*	CH	ACI28281
		Acinetobacter calcoaceticus	CH	FJ194460
	OXA-165 to OXA-171	*A. baumannii*	TH	HM488986 to HM488992
	OXA-422, OXA-423	*A. baumannii*	CH	KM433671, KM433672
	OXA-40 like			
	OXA-40	*A. baumannii*	IN, TW	15,48
		A. nosocomialis	TW	15
	OXA-72	*A. baumannii*	TH, TW	AY739646,[65]
		A. pittii	CH	103
	OXA-58 like			
	OXA-58	*A. baumannii*	AU, CH, IN, JP, KO, SP, TW	28,48,52,53,61,88,114
		A. nosocomialis	SP, TW,	52,61
		A. pittii	JP, SP, TW	40,52,107
	OXA-96	*A. baumannii*	SP	51
	OXA-420	*A. baumannii*	NP	AB983359
	OXA-143 like			
	OXA-182	*A. baumannii*	KO	49
MBLs	IMP like			
	IMP-1	*A. baumannii*	IN, JP, KO, TW	43,57,73,74
		A. nosocomialis	JP, KO	49,53
		A. pittii	JP, KO, TW	40,49,53
		A. calcoaceticus	JP	53
		Acinetobacter lwoffii	JP	53
	IMP-2	*A. baumannii*	IN	73
	IMP-4	*A. baumannii*	HK	14
		A. nosocomialis	HK, SP	14,51
		A. pittii	HK, SP	14,51
		Acinetobacter junii	AU	76
	IMP-8	*A. baumannii*	TW	59
		A. pittii	TW	40
	IMP-11	*A. pittii*	JP	107
	IMP-14	*A. baumannii*	TH	46
	IMP-19	*A. baumannii*	JP	108
		Acinetobacter bereziniae	JP	107
		Acinetobacter johnsonii	JP	108
		A. junii	JP	108
		A. nosocomialis	JP	107
		A. pittii	JP	107
	VIM like			
	VIM-1	*A. baumannii*	IN	73
		A. lwoffii	IN	3
	VIM-2	*A. baumannii*	IN, KO, TW	49,59,73
		A. nosocomialis	KO	88
		A. pittii	KO	49
	VIM-3	*A. baumannii*	TW	59
	VIM-11	*A. baumannii*	TW	59
		A. haemolyticus	TW	59
	SIM-1	*A. baumannii*	KO	58
		A. bereziniae	KO	49
		Acinetobacter baylyi	CH	115
	NDM-1	*A. baumannii*	BA, CH, IN, JP, PK	12,31,41,47,70
		A. johnsonii	CH	117
		A. junii	CH	116
		A. lwoffii	CH	38
		A. pittii	CH	109

AU, Australia; BA, Bangladesh; CH, China; JP, Japan; HK, Hong Kong; IN, India; KO, South Korea; NP, Nepal; PK, Pakistan; SP, Singapore; TH, Thailand; TW, Taiwan; VT, Vietnam.

MBL, metallo-β-lactamases.

environment have yet to be determined. The genome-wide approach may provide such insight for prevention and control of further *Acinetobacter* spp. transmission.

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Disclosure Statement

D.L.P. has been on the advisory boards for Merck, AstraZeneca, Cubist, Bayer, and Pfizer.

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1.3 Genome and resistance island of *A. baumannii*

The sizes of *Acinetobacter* spp. genomes varied from 4.9 Mb observed in *A. bereziniae* (GenBank accession no. APQG00000000) to 2.7 Mb in *Acinetobacter nectaris* (GenBank accession no. AYER00000000). The highest GC content at 41.5% was identified in the genome of *Acinetobacter brisouii* while the lowest GC content was 36.6% in *A. nectaris* [12].

Amongst *A. baumannii* genomes, the range of estimated size is 3.7 – 4.2 Mb with GC content approximately at 39% [13]. Multiple genomic studies demonstrated the great diversity amongst *Acinetobacter* genus and with *A. baumannii* [7, 12, 14]. The comparative genomic approach of multidrug-resistant *A. baumannii* strains and fully susceptible strains identified a genomic island containing a large cluster of antimicrobial and heavy metal resistance genes and was designated as "AbaR" [15]. AbaR usually located in a specific chromosomal ATPase gene, *comM*. [16].

The first and the largest cluster, AbaR1, was recovered from French strain AYE, which belongs to IC1 [15]. This 86.2-kb resistance island is a large composite transposon that carries horizontally transferred genes conferring resistance to a broad range of antimicrobial families. Later on, AbaR3 and AbaR5 which are related to AbaR1 were discovered in *A. baumannii* of IC1 [7, 17]. AbaR3 is largely a subset of AbaR1 at 49 kb; on the other hand, AbaR5 is most similar to AbaR3 and shares the same general structure. The *catA1* and *bla*$_{\text{TEM}}$ genes found in AbaR3 are not present in AbaR5, due to an IS*26*-induced deletion of 6.7-kb region in AbaR5. AbaR1 also lacks the *bla*$_{\text{TEM}}$ genes found in AbaR3 due to a deletion caused by the same IS*26* [16, 18]. Studies on various isolates belonged to IC1 identified several new variant of AbaR3 and showed that AbaR3 was the original form of resistance island of IC1horbouring Tn*6019* and Tn*6018* as a backbone [16, 18-21].

AbaR2 identified in an IC2 strain ACICU is approximately 19.5 kb and lacks arsenic, mercury and tetracycline resistance operons on the 5' end compared to which is found in standard AbaR [22]. Therefore, this island plays a significant but less dominant role in resistance to the clinically important classes of antimicrobials including carbapenems. AbaR4 was firstly discovered in susceptible strain ATCC 17978 and related to resistance islands found in IC2 strains [7, 16]. The backbone of AbaR4-type of IC2 strain, Tn*6022*, composed of transposition module (*tni*), *uspA* (universal stress protein encoding gene) and *sup* (sulphate permease) [23]. AbaR4-type was also identified as a common location for Tn*2006* containing *bla*$_{\text{OXA-23}}$, which conferred carbapenem resistance phenotype [7, 24, 25].

1.4 Hospital adaptiveness of *A. baumannii*

The rapid emergence and global dissemination of distinct *A. baumannii* clonal lineages as a major nosocomial pathogen is remarkable and emphasizes its successful adaption to the present day hospital environment. Little is known concerning the origin of these clonal lineages. It is possible that each distinct cluster has originated from a different location and then spread into a new niche. The spread may be due to occupational transmission, such as cross-transmission from colonised or infected patients via the hands of health-care workers [26, 27].

The role of the hospital environment as a reservoir for *A. baumannii* is supported by the fact that this organism can be recovered from patients and various hospital environmental sources during outbreaks [28]. A number of studies show that particular strains can be isolated from the same hospital during a long period of time [28-30]. The ability to survive under desiccative conditions as well as resistance to disinfectants and antimicrobials demonstrate how well *A. baumannii* can adapt and lead to long-term persistence in the hospital environment. The following aspects that may contribute to its persistence will be discussed and putative genes for hospital adaptiveness are proposed in Table 1.

Table 1. Putative genes for hospital adaptiveness.

Name of gene or protein	Function	Reference
csuC and *csuE*	• Secretion and pili assembly • Biofilm formation	[31]
bla$_{\text{PER-1}}$	• β-lactamase production • Associated with cell adhesiveness	[32, 33]
Bap	• Intercellular adhesion • Biofilm maturation	[34]
pga	• PNAG synthesis	[35]

1.4.1 *Biofilm formation*

Biofilm formation is hypothesized to mediate the prolonged survival of *Acinetobacter* in healthcare settings [36, 37]. *A. baumannii* clinical isolates can survive following long periods of desiccation and can form biofilm on biotic and abiotic – nonliving surfaces. However, no statistically significant difference between the response to drying, measured by the survival time on glass coverslips, of outbreak strains versus those of sporadic strains was found by Jawad *et al.* [36]. A number of studies showed that the ability to form biofilm is common among unrelated clinical isolates of *A. baumannii* [33, 38], particularly related to device-associated infections [38]. Additionally, correlations between the biofilm-forming property and broad-spectrum antimicrobial resistance phenotypes in some clinical isolates was reported [33].

The high capacity for biofilm production amongst extended-spectrum β-lactamase (ESBL) producing *A. baumannii* isolates, *bla*$_{\text{PER-1}}$, was observed [33]. Lee *et al.* confirmed this by elucidating that cell adhesiveness and biofilm formation on the surface of polystyrene, a polymer that is widely used in many kinds of medical devices, were significantly higher in isolates harbouring the *bla*$_{\text{PER-1}}$ gene as compared with isolates without this gene [32]. Nevertheless, some studies showed that there was no correlation between *bla*$_{\text{PER-1}}$ positive isolates and biofilm producers [33, 39]. Therefore, it is considered that the presence of *bla*$_{\text{PER-1}}$ is more crucial for cell adhesion than biofilm formation. The reasons for this have not yet been fully explored since there is no data available on the knockout of *bla*$_{\text{PER-1}}$ in *A. baumannii*.

Many regulatory networks are believed to be associated with the expression of the biofilm phenotype, such as cellular appendages, adhesions and cell density-sensing molecules. The somatic pili that mediate the initial steps of biofilm formation on polystyrene found in the *A. baumannii* 19606 strain are the product of chaperon-usher secretion system called CsuA/BABCDE [31]. Similar operons were also discovered in the genome of strains AYE [40] , ACICU [22], AB0057 and 17978 [41]. On the contrary, no similar loci coding for this secretion system were located in the genome of *A. baumannii* SDF, isolated from body lice collected from homeless people and *Acinetobacter* sp. ADP1. Additionally, the inactivation of the chaperone-encoding genes, *csuC* and *csuE* led to an obliteration of pili production and biofilm formation [31]. The expression of this operon is regulated by a two-component system BfmRS containing a sensor kinase encoded by *bfmS* and a response regulator encoded by *bfmR* [42].

In the *A. baumannii* clinical strain 307-0294, biofilm-associated protein (Bap) which was first characterized in *S. aureus*, was found to act in intercellular adhesion which then supported biofilm maturation [34]. Another study from Choi *et al.* showed that *A. baumannii* clinical isolates contained

pga locus encoding protein which synthesizes surface polysaccharide poly-β-(1-6)-N-acetyl glucosamine (PNAG). Deletion of this locus resulted in loss of the strong biofilm phenotype, which was restored by complementation [35].

1.4.2 *Desiccation tolerance*

Acinetobacter spp. is found to survive far better on fingertips or on dry surfaces when tested under simulated hospital environmental conditions than other genera of Gram negative bacilli [43, 44]. It has been shown that *A. baumannii* survives desiccation beyond 30 days and much better than other *Acinetobacter* spp. such as *A. johnsonii*, *A. junii* and *A. lwoffii* [43, 44]. However, skin carriage of *A. baumannii* is very rare, whilst *A. johnsonii*, *A. lwoffii* and *A. radioresistens* predominate on both patient and healthy human skin [45, 46]. Although there was no statistically significant difference between the survival times of sporadic strains and outbreak strains of *A. baumannii* [36], desiccation tolerance may contribute to the propensity to cause prolonged nosocomial infection outbreaks and may explain why certain strains are able to establish themselves in hospital environment while others are only sporadically isolated.

1.4.3 *Biocide resistance*

The co-resistance to antimicrobials and biocides, including disinfectants and antiseptics, may contribute to the selection of drug-resistant strains and to epidemic spread within the hospital [47]. Correlation between decreased susceptibility to disinfectants and antimicrobial resistance have been found in various organisms such as *Pseudomonas aeruginosa*, *Proteus* spp., *Providencia* spp., *Serratia marcescens*, MRSA and vancomycin-resistant enterococci [48-51]. A similar hypothesis has also been proposed for *A. baumannii* [52, 53]. An *in vitro* study using currently used disinfectants (propenol, combination of 1-propanol, 2-propanol and mecetronium ethylsulphate, PVP-iodine, tricolsan and chlorohexidine) showed no significant differences in susceptibility between ten outbreak-related and ten sporadic strains. However, a relevant number of viable bacteria remained if contact times were less than 30 seconds or diluted agents were used [53]. Although resistance to disinfectants is probably not a major factor involved in the epidemic spread of *A. baumannii*, slightly deviated conditions from recommended procedures leading to decreased concentrations or exposure times may be important for nosocomial cross-contamination and help promote viability of *A. baumannii* in hospital environments.

1.5 Virulence and pathogenicity of *A. baumannii*

Despite convincing evidence supporting *A. baumannii* as a successful nosocomial pathogen, the knowledge of the factors determining epidemicity, virulence factors and pathogenicity is still not well defined. However, the ability of *Acinetobacter* spp. to adhere to epithelial cells, produce enzymes and toxins and possess anti-phagocytic surface components are considered to be significant virulence mechanisms of this genus [54]. Moreover, comparative genomic studies between *A. baumannii* and the environmental *A. baylyi* (non-pathogenic) revealed potential virulence genes in *A. baumannii* involved in pili biogenesis, iron regulation and quorum sensing [55]. More recent studies suggested that type VI secretion systems (T6SS) responsible for eliciting immune response in eukaryotic cell may play a role in inter-bacterial and host-bacterial interactions in *A. baumannii* [56, 57].

1.5.1 *Colonization and adherence*

Its ability to cause colonization on human skin and inanimate surfaces also has an important role in infection, epidemic spread and environmental persistence [2]. As adherence of microorganisms to host cells is the initial step of the colonization process, an *in vitro* study of *A. baumannii* adherence was performed by Lee *et al* [58]. Their study showed that *A. baumannii* could adhere to human bronchial epithelial cells. Although strains of IC2 had a relatively high capacity for adhering to these cells compared to IC1 strains, there was no significant correlation of the outbreak-associated strains with the ratio of infected cells.

Following adhesion, *A. baumannii* is able to invade and promote the apoptosis of eukaryotic cells [59]. A major surface protein, outer membrane A of *A. baumannii* has been demonstrated as a potential virulence factor in inducing cell death through mitochondrial and nuclear targeting [59, 60]. Purified Omp38 also induced apoptosis of human bronchial epithelial cells and human monocytes. Apoptosis of epithelial cells may disrupt the mucosal lining and allow the bacterial access or their products into the deep tissues [61].

1.5.2 *Surface polysaccharide*

Surface polysaccharide, capsular (K antigen) and lipopolysaccharide (LPS) carrying O-antigen, are known as core virulence factors in many Gram-negative bacteria. It has been demonstrated that surface polysaccharide promoted motility and acts as a barrier for bactericidal activity [62]. K1 antigen in *A. baumannii* showed an ability to improve growth significantly in human ascites fluid, human serum resistance and survival in a rat soft-tissue infection mode [63]. A recent study in *A. baumannii* genomes revealed that *A. baumannii* lacked a ligase encoding gene *waaL* required for

addition of O-antigen to lipooligosaccharide (LOS) resulting in failure to form LPS [64]. In Gram-negative bacteria, these regions responsible for capsule and the outer core (OC) of LOS are variable and cause antigen heterogeneity [65, 66].

1.5.3 *Iron regulation*

The ability of *A. baumannii* to grow under iron-deficient conditions is known to be associated with invasiveness. *A. baumannii* can express a variety of molecules regarding iron acquisition, including the iron-regulated catechol siderophore compounds, catechol-hydroxamate siderophore - acinitobactin and also a hemin utilisation system [67]. Furthermore, there is a wide variability within members of the same set of isolates or their nosocomial origin in the expression of molecules involved in iron regulation [68].

1.5.4 *Quorum sensing*

Quorum sensing (QS), a known autoinducer-receptor mechanism, is one type of bacteria cell-cell communication. This mechanism plays a role in the production of virulence factors, motility, nodulation, sporulation, plasmid transfer, antimicrobial production, as well as biofilm formation [69, 70]. There was an association between Acyl Homoserine Lactones (AHL) mediated QS and phenotypes that benefit the AHL-producing community, including biofilm formation [71]. Most *Acinetobacter* spp. strains produce more than one AHL. However, none of the AHL signals could be specifically assigned to a particular species of the *Acinetobacter* spp. [72]. *Acinetobacter* spp. quorum signals are not homogenously distributed, thus it is difficult to distinguish between virulent and non-virulent strains in terms of quorum sensing signals. Quorum sensing genes, *aba*I and *aba*R of *Acinetobacter* spp., were acquired horizontally from *Halothiobacillus neapolitanus* [73]. Communication among bacteria pertaining to cell density is integral to maturation of *A. baumannii* biofilm [74]. Mutation in *aba*I which produces the AHL molecule resulted in reduction in biofilm when compared with its isogenic parental strain [75]. Exogenous addition of purified *Acinetobacter* acyl homoserine lactone restored biofilm maturation in an *aba*I mutant [76].

1.6 Aims

ACB complex has its own challenge for the species identification with *A. baumannii* recognised as the most important nosocomial pathogen [77]. In early 1990s, carbapenem resistance was first recognised in *A. baumannii* (CRAB) and the prevalence of CRAB has drastically increased worldwide [78]. Apart from carbapenem resistance in *A. baumannii*, the co-resistance to broad-spectrum aminoglycosides and other non-β-lactams restricts the treatment of this organism to very few antimicrobial options [79, 80].

Carbapenem resistance imparted by oxacillinases in *Acinetobacter* spp. is a growing problem in many regions of the world [24]. However, there are many gaps in the current literature regarding specific resistance to carbapenems and the genomes of ACB complex in Asia and Oceania. A comprehensive review of the epidemiology and mechanisms of carbapenem resistance within *Acinetobacter* spp. in this thesis was used to provide the basic understanding of the most recent studies in *A. baumannii* in Asia and Oceania [81]. Prior to studies in this thesis, there was limited description of the genomes of *Acinetobacter* spp. not only from South East Asia, but also from Asia and Oceania in general.

Over the past two decades, characterisation by molecular epidemiology has been used to understand the types of dominant clones and worldwide distribution of *A. baumannii* clones [11, 82]. A successful clone of carbapenem-resistant *A. baumannii* IC2 has been the major driving force for the spread of carbapenemase among *A. baumannii* [11]. Little attention has been given to non-*baumannii Acinetobacter*. In particular to Thailand, the molecular and genome description of *Acinetobacter* spp. was limited [83-86].

The specific aims of the serial experiments described in this thesis are as follows:

1. To determine the molecular epidemiology of *A. baumannii*, primarily from Thailand.
2. To detect species specific intrinsic oxacillinases and description of non-*baumannii Acinetobacter*, in particular, *A. pittii*.
3. To describe the genome of *A. baumannii* IC2 isolates from Thailand and compare these genome data with *A. baumannii* IC2 isolates from Japan, Malaysia and Singapore.

Chapter 2. Detection of species specific intrinsic oxacillinases and characterisation of pathogenic non-*baumannii Acinetobacter* spp.

2.1 Synopsis

Acinetobacter is a complex non-fermentative gram-negative bacteria genus comprising of more than 40 species [77]. Although the most common *Acinetobacter* species involved in hospital infections is *A. baumannii*, other species, such as *A. pittii, A. nosocomialis, A. haemolyticus, A. johnsonii, A. lwoffii and A. ursingii,* are sporadically recovered from clinical specimens and involved in nosocomial infections [87].

Due to high similarities of phenotypic and genotypic characteristics, it is difficult to differentiate *Acinetobacter* spp. into species level [88]. Available semi-automated systems used in routine diagnostic laboratories, such as API-20NE and Vitek 2 system are not be able to distinguish amongst Acb complex while phenotypic and genotypic methods, such as DNA – DNA hybridization and amplified rRNA gene restriction analysis (ARDRA) are laborious [89, 90].

In this Chapter, two main research studies performed on non- *baumannii Acinetobacter* spp. were included. The first paper, we detected naturally occurring oxacillinases that are species specific using a multiplex PCR and sought a use of this method to aid in species identification. In addition, thirty novel OXA variants were discovered amongst *Acinetobacter* isolates used in this study. The second paper provides the first whole genome sequence of IMP and OXA-58-like-carrying *A. pittii* ST119 in Australia. This strain harboured several classes of antimicrobial resistance genes as well as a novel bla_{OXA} variant, $bla_{\text{OXA-421}}$. Further, the investigation of acquired OXA-type carbapenemases in *Acinetobacter* non-*baumannii* isolates collected worldwide was demonstrated in the Appendix A2.4.

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Multiplex PCR to detect the genes encoding naturally occurring oxacillinases in *Acinetobacter* spp.

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Objectives: Bacteria of the genus *Acinetobacter* are increasingly being isolated in hospitals and are recognized as emerging nosocomial pathogens. Species identification is difficult and there is a need for simple molecular methods to differentiate between the species. Naturally occurring oxacillinase genes (bla_{OXA}) have been identified in several *Acinetobacter* species and their detection by PCR can aid in species identification. The aim of this study was to develop a multiplex PCR to identify intrinsic bla_{OXA} genes (i.e. $bla_{OXA\text{-}134\text{-}like}$, $bla_{OXA\text{-}211\text{-}like}$, $bla_{OXA\text{-}213\text{-}like}$, $bla_{OXA\text{-}214\text{-}like}$ and $bla_{OXA\text{-}228\text{-}like}$) from *Acinetobacter* spp. for use as a tool for rapid species identification.

Methods: Primers were designed to selectively amplify internal fragments of intrinsic bla_{OXA} from *Acinetobacter lwoffii/Acinetobacter schindleri* ($bla_{OXA\text{-}134\text{-}like}$), *Acinetobacter johnsonii* ($bla_{OXA\text{-}211\text{-}like}$), *Acinetobacter calcoaceticus* ($bla_{OXA\text{-}213\text{-}like}$), *Acinetobacter haemolyticus* ($bla_{OXA\text{-}214\text{-}like}$) and *Acinetobacter bereziniae* ($bla_{OXA\text{-}228\text{-}like}$). Multiplex PCR was performed in a total of 100 *Acinetobacter* isolates. Flanking primers were designed for each bla_{OXA} subgroup and products were sequenced.

Results: All *A. lwoffii*, *A. schindleri*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus* and *A. bereziniae* isolates were positive for their species-specific amplicons while other *Acinetobacter* species were negative. Thirty bla_{OXA} novel variants were identified; the majority of these (21/30) were from *A. calcoaceticus*. IS*Aba11* was found upstream of $bla_{OXA\text{-}214}$ in four *A. haemolyticus* isolates, but was not associated with carbapenem resistance.

Conclusions: This multiplex PCR specifically detected each of the five different bla_{OXA} subgroups. Therefore, this method has the potential to aid in the identification of these species and monitor the spread of these genes into other *Acinetobacter* species.

Keywords: species identification, carbapenemases, intrinsic OXA

Introduction

Bacteria of the genus *Acinetobacter* are recognized as pathogens that frequently cause nosocomial infections.[1] Although the most common *Acinetobacter* species involved in hospital infections are those belonging to the *Acinetobacter baumannii* group (*A. baumannii*, *Acinetobacter nosocomialis* and *Acinetobacter pittii*), other species, such as *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter ursingii*, are sporadically recovered from clinical specimens and involved in nosocomial infections.[2] *Acinetobacter calcoaceticus*, although an environmental organism, is often found as a colonizer and is frequently misidentified as *A. baumannii*.[3]

Species identification of *Acinetobacter* in routine diagnostic laboratories is difficult and these organisms are frequently misidentified.[4] Phenotypic and molecular techniques for species identification, such as DNA–DNA hybridization and amplified rRNA gene restriction analysis ('ARDRA'), are laborious and difficult to interpret, while semi-automated systems, such as API-20NE and Vitek 2, identify many *Acinetobacter* as *A. calcoaceticus–A. baumannii* (Acb) complex.[5,6] Sequencing methods based on the *rpoB* gene, its flanking spacer regions and the 16S–23S rRNA gene spacer region are reliable for species identification, but it is unlikely that these sequencing techniques will be used routinely.[7,8] More recently, a rapid PCR-based method for detecting *gyrB* genes was described by Higgins *et al.*[9,10] This tool can also be utilized for species identification amongst bacteria of the Acb complex.

Genes encoding naturally occurring oxacillinases (OXAs) have been identified in several *Acinetobacter* species, such as $bla_{OXA\text{-}23\text{-}like}$ (*Acinetobacter radioresistens*), $bla_{OXA\text{-}51\text{-}like}$ (*A. baumannii*), $bla_{OXA\text{-}134\text{-}like}$ (*A. lwoffii/Acinetobacter schindleri*), $bla_{OXA\text{-}211\text{-}like}$ (*A. johnsonii*), $bla_{OXA\text{-}213\text{-}like}$ (*A. calcoaceticus*),

$bla_{OXA-214-like}$ (*A. haemolyticus*) and $bla_{OXA-228-like}$ (*Acinetobacter bereziniae*).[11–15] The PCR detection of $bla_{OXA-51-like}$ has been proposed as a method to identify *A. baumannii*.[16] Amongst other species, the detection of $bla_{OXA-134-like}$ has been shown to be an alternative for rapid identification of *A. lwoffii* and *A. schindleri*.[17] This suggests that the detection of the genes harbouring intrinsic OXAs may be applied as a tool to identify these *Acinetobacter* species.

The aim of this study was to develop and evaluate a multiplex PCR assay to detect five species-specific gene subgroups encoding the intrinsic OXA-134-like, OXA-211-like, OXA-213-like, OXA-214-like and OXA-228-like as a potential method to rapidly identify *A. lwoffii/A. schindleri*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus* and *A. bereziniae*, respectively.

Materials and methods

Bacterial isolates

A total of 100 clinical isolates and type and reference strains were included. *A. calcoaceticus* (25), *A. bereziniae* (9), *A. haemolyticus* (5), *A. johnsonii* (11), *A. lwoffii* (10) and *A. schindleri* (2) were selected from our own clinical culture collection.[9,18] Clinical isolates of the species *Acinetobacter junii* (2), *A. pittii* (2), *A. baumannii* (8), *A. nosocomialis* (4), *Acinetobacter beijerinckii* (5), *Acinetobacter guillouiae* (2), *A. radioresistens* (2), *A. ursingii* (2) and the unnamed *Acinetobacter* genomic species 14 (2) were also included as controls. Species identification had been performed previously and was confirmed for all isolates by partial *rpoB* sequencing.[8]

The *A. baumannii* isolates were chosen to represent a broad range of $bla_{OXA-51-like}$ variants (OXA-65, -66, -95, -223 and -241) and acquired bla_{OXA} (OXA-23, -40, -58, -72, -143, -235 and -236). In addition, the following type or reference strains were used: *A. baumannii* ATCC 19606^T, *A. bereziniae* ATCC 17924^T, *A. calcoaceticus* ATCC 23055^T, *A. johnsonii* ATCC 17909^T, *A. junii* ATCC 17908^T, *A. lwoffii* NCTC 5866, *A. radioresistens* SEIP 12.81, *A. pittii* ATCC 19004^T and *Acinetobacter* genomic species 6 ATCC 17979^T.

Primer design

All primers used in this study were designed using Primer3 software (http://bioinfo.ut.ee/primer3/). The DNA sequences of each bla_{OXA} subgroup available in GenBank and those from whole genome sequences (Table S1, available as Supplementary data at *JAC* Online) were aligned. Consensus regions of each subgroup were used to design five pairs of primers corresponding to their subgroups (Table 1). To aid identification in a multiplex format, the sizes of PCR products were designed so that there was ~100 bp difference between each subgroup.

Multiplex PCR

Multiplex PCR was undertaken in a final volume of 25 μL using *Taq* PCR Master Mix (Qiagen, Hilden, Germany) with a final concentration of 0.2 μM for each primer. Template DNA for PCR was isolated from an agar plate; a 1 μL loopful of a colony was suspended in 100 μL of PCR-grade water, boiled for 10 min, snap-cooled and briefly centrifuged. The amplification conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 1 min, and final extension for 10 min. PCR products were analysed on agarose 1.2% (w/v) gels, stained with ethidium bromide and visualized on a UV transilluminator.

DNA sequencing and accession numbers

Flanking primers of each bla_{OXA} subgroup were designed (Table 1) based on available genome sequences in the NCBI database. PCR amplification was performed following the same conditions as the multiplex PCR using Phusion hot-start high-fidelity DNA polymerase (Thermo Fisher Scientific, Schwerte, Germany) and products were sequenced in both directions. Novel sequences were assigned numbers by the Lahey β-lactamase database (http://www.lahey.org/studies/) and submitted to EMBL/GenBank under accession numbers KF203096–KF203109 ($bla_{OXA322-335}$), KF297577–KF297585 ($bla_{OXA348-356}$), KF421160–KF421163 ($bla_{OXA357-360}$) and KF460531–KF460533 ($bla_{OXA361-363}$).

Results

All primers used in this study are listed in Table 1. Sequencing *rpoB* variable regions 1 and 2 confirmed the species identity of the isolates. The bla_{OXA} primers were initially tested as a multiplex against all isolates in order to evaluate their specificity and sensitivity. When an isolate gave a positive PCR product, the bla_{OXA} gene was re-amplified using the flanking primers and sequenced. This strategy allowed us to design new primers where appropriate. For example, the original primers designed to amplify $bla_{OXA-134-like}$ (*A. lwoffii/A. schindleri*) also amplified $bla_{OXA-228-like}$ (*A. bereziniae*). In this case, new primers were designed and tested as a singleplex to determine the optimum conditions before multiplexing (data not shown).

The multiplex PCR assay using five pairs of primers amplified the intrinsic bla_{OXA} genes of each subgroup as predicted. The PCR products, ranging from 158 to 693 bp, were easily separated and visualized on an agarose gel (Figure 1). All *A. lwoffii/A. schindleri*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus* and *A. bereziniae* isolates were positive for their species-specific amplicons whilst all isolates of the other *Acinetobacter* species were negative (including *A. baumannii* isolates harbouring other intrinsic and acquired bla_{OXA}s). A modification of this multiplex, addition of *rpoB* primers 696F and 1598R as internal control as previously described, was also evaluated.[17] The high annealing temperature we used for the multiplex led to a reduced *rpoB* amplicon concentration, especially in isolates that were positive for bla_{OXA} (data not shown). However, species that are negative for bla_{OXA} could be identified by sequencing the *rpoB* PCR product.

Sequencing bla_{OXA} identified 30 new variants (Table S2, available as Supplementary data at *JAC* Online). The majority of these were from *A. calcoaceticus*, where we found 23/26 isolates with a novel variant. Twenty-one new variants of $bla_{OXA-213}$ were identified. The range of amino acid substitutions encoded by these genes when compared with $bla_{OXA-213}$ was from 23 (97.2% similarity) to 59 (92.8% similarity). A premature stop codon was identified in three isolates: *A. calcoaceticus* (2) and *A. johnsonii* (1). Flanking primers (G56/G57) gave an ~3 kb product size in four *A. haemolyticus* isolates. Sequencing of the amplicons identified an IS element upstream of $bla_{OXA-214}$ with a BLAST match to IS*Aba11* (97.8%). The presence of IS*Aba11* was not associated with imipenem or meropenem resistance by Etest using EUCAST guidelines for interpretation. IS*Aba11* is an emerging IS family encoding transposases.[19] This IS element is not commonly responsible for carbapenem resistance in *Acinetobacter* spp. However, IS*Aba11* was reported to be related to high-level colistin resistance in *A. baumannii*.[20] Additionally there is a report that the bla_{NDM-1} region in a plasmid found in *A. pittii* isolates was flanked by IS*Aba11* and IS*Aba125*.[21] We were unable to sequence bla_{OXA} from four isolates—*A. lwoffii* (2), *A. johnsonii* (1) and *A. schindleri* (1)—despite redesigning primers several times. Sequence analysis based on published and our own data revealed the DNA flanking these

Table 1. Primers used in this study

Function	Target	Primer	Sequence (5′–3′)	Amplicon (bp)	Species
1	$bla_{OXA\text{-}134\text{-}like}$	G50	CAGGAAGTACAACGCATCCA	158	*A. lwoffii*, *A. schindleri*
		G51	TGCTGGACTTGAGGATCAAA		
	$bla_{OXA\text{-}211\text{-}like}$	G24	CAACCAGCACCGAGGTATTT	244	*A. johnsonii*
		G25	TGAACAGGCGTAATTTGCAG		
	$bla_{OXA\text{-}213\text{-}like}$	G26	TTTCCTGATTGGGAAAAGGA	401	*A. calcoaceticus*
		G27	GCGACAATTTCTCCTTGTGG		
	$bla_{OXA\text{-}214\text{-}like}$	G48	TCTGAATCGTGCCAAAACTG	518	*A. haemolyticus*
		G49	TTCCGTTCGGATCTTCAATC		
	$bla_{OXA\text{-}228\text{-}like}$	G30	GTTTGGCATTTTCAGGTTGTG	693	*A. bereziniae*
		G31	TTAACGCAAATGCAGTCACC		
2	flanking $bla_{OXA\text{-}134\text{-}like}$	V2_F	GGCGAAGGTCAATCTCAAAA	1150	*A. lwoffii*
		V2_R	CGAGCAGACAGAGCAGAAGA		
	flanking $bla_{OXA\text{-}211\text{-}like}$	G34	GATGGCGTTGTAGATGCTGA	1281	*A. johnsonii*
		G35	AAAGCAAACAAGAGACTTTGACG		
	flanking $bla_{OXA\text{-}213\text{-}like}$	G36	TTCCTTTGTTTATGCTTTCCTTTT	1103	*A. calcoaceticus*
		G37	AGTGGCTTGATGCTGCTTTT		
	flanking $bla_{OXA\text{-}214\text{-}like}$	G56	GTTTTCTAGCTCGGCTTTCC	1132	*A. haemolyticus*
		G57	TCAGCCATCAAGCCACATAC		
	flanking $bla_{OXA\text{-}134\text{-}like}$	G60	TCGTATTTTCAGGCAAAGCTG	1090	*A. schindleri*
		G61	AAGCGCGTATCAAAAGGATG		
	flanking $bla_{OXA\text{-}228\text{-}like}$	F5	GCTAAAGTTTCTGCTGAGGA	1151	*A. bereziniae*
		F6	CCAGTTACCCCCAATAAACT		

Function 1, multiplex PCR primers; Function 2, sequencing primers.

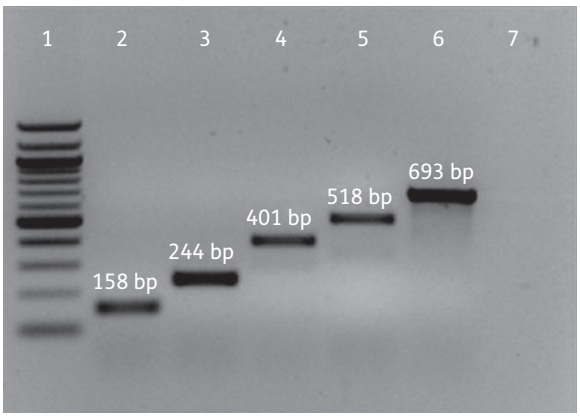

Figure 1. Example of an agarose gel showing *Acinetobacter* isolates for which the species were determined by multiplex PCR. Lane 1, 100 bp marker; lane 2, *A. lwoffii/A. schindleri*; lane 3, *A. johnsonii*; lane 4, *A. calcoaceticus*; lane 5, *A. haemolyticus*; lane 6, *A. bereziniae*; lane 7, negative control.

bla_{OXA}s to be very variable and a clear consensus was not possible (data not shown). However, these four isolates had their species identity confirmed by *rpoB* sequencing and were positive by multiplex PCR for their intrinsic bla_{OXA}.

A phylogenetic tree of intrinsic bla_{OXA}s both used and identified in this study was constructed (Figure 2). Each subgroup clustered in one of five distinct branches. The results show that *A. lwoffii* and *A. schindleri* intrinsic bla_{OXA}s were very similar and clustered together in one main branch. Therefore, it was not possible to differentiate between them in the current multiplex PCR format. To further identify these species, an alternative method, such as *rpoB* sequencing, should be applied.[8]

Discussion

The results of the multiplex PCR assay for genes encoding intrinsic species-specific bla_{OXA}s showed that the assay was well correlated between OXA subgroup and predicted amplicon size. There was no PCR product amongst the other *Acinetobacter* species tested, suggesting the assay is specific and sensitive enough for detection of intrinsic bla_{OXA}s. Therefore, this method has potential to be used as an alternative, rapid tool to confirm species identification of *A. lwoffii/schindleri*, *A. johnsonii*, *A. calcoaceticus*, *A. haemolyticus* and *A. bereziniae*.

Intrinsic bla_{OXA}s are capable of conferring carbapenem resistance when overexpressed. For example, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}228\text{-}like}$ and $bla_{OXA\text{-}23\text{-}like}$, when adjacent to insertion elements, cause carbapenem resistance.[22–24] Some of these intrinsic genes have also been detected in other species; $bla_{OXA\text{-}23\text{-}like}$ has become the most commonly acquired carbapenem resistance determinant in *A. baumannii* and is also found in other *Acinetobacter* species, and $bla_{OXA\text{-}51\text{-}like}$ was reported in *A. nosocomialis*.[25–27] In these instances mobilization was associated with IS*Aba1*, which encodes not only a transposase but also a promoter, leading to overexpression of the OXA and resulting in carbapenem resistance.

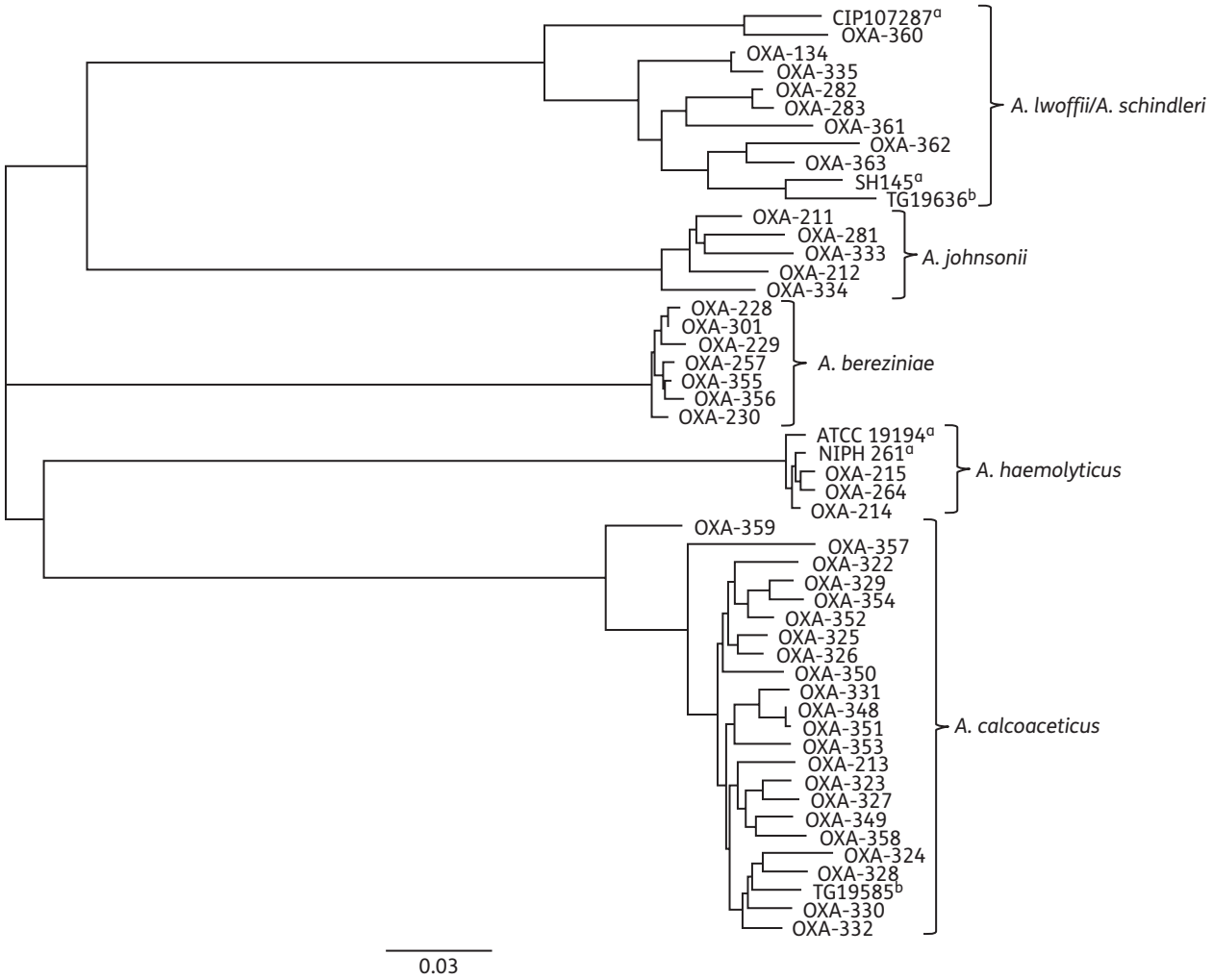

Figure 2. Dendrogram of intrinsic OXAs identified and used in this study. The tree was constructed using Geneious version 6.1 created by Biomatters, available from http://www.geneious.com. [a]Nucleotide sequence of bla_{OXA} acquired from the draft whole genome sequence in the NCBI database where an OXA number has not been assigned. The locus tags for these bla_{OXA} genes are: F955_00114, *A. schindleri* CIP107287; HMPREF0017_02813, *A. lwoffii* SH145; HMP0015_3373, *A. haemolyticus* ATCC 19194; F926_00636, *A. haemolyticus* NIPH 261. [b]Unannotated raw genome data of bla_{OXA}: *A. lwoffii* TG19636; *A. calcoaceticus* TG19585.

Therefore, detection of bla_{OXA} should not be the only identification method because there are no data on the distribution of these bla_{OXA}s into other species. However, in the present study we have not detected these bla_{OXA}s in other *Acinetobacter* species that are not known to intrinsically harbour these genes.

We identified IS*Aba11* located upstream of $bla_{OXA\text{-}214}$ in four *A. haemolyticus* isolates. Even though our findings showed that IS*Aba11* was not associated with carbapenem resistance in *A. haemolyticus*, this IS element is also found in *A. baumannii*[19,20] and may potentially help facilitate mobilization of $bla_{OXA\text{-}214}$. Hence the multiplex PCR we describe can also be used to screen isolates to determine whether there is dissemination of these bla_{OXA}s into *A. baumannii* when there are no other carbapenem resistance determinants detected.

Large variation in *A. calcoaceticus* bla_{OXA} is unsurprising given that this species is widely distributed in the environment. Conversely, *A. lwoffii*, *A. johnsonii*, *A. haemolyticus* and *A. bereziniae*, which are mostly recovered from human clinical specimens, show less variation of their bla_{OXA}s. It is also worth noting that the *A. baumannii* intrinsic $bla_{OXA\text{-}51\text{-}like}$ also shows a lot of variation, but phylogenetic analysis of this species shows that bla_{OXA} is related to epidemiological grouping and carbapenem resistance.[28] The variation in *A. calcoaceticus* bla_{OXA} thus warrants further study.

In conclusion, this multiplex PCR specifically detects each of the five different bla_{OXA} subgroups that are associated with a distinct *Acinetobacter* species or, in the case of $bla_{OXA134\text{-}like}$, two species. Therefore, this method has the potential to aid in the identification of these species and monitor the spread of these genes into other *Acinetobacter* species.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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A Case of IMP-4-, OXA-421-, OXA-96-, and CARB-2-Producing *Acinetobacter pittii* Sequence Type 119 in Australia

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An IMP-4-producing *Acinetobacter pittii* strain coproducing oxacillinases was isolated from a leg wound of a 67-year-old female patient. Identification to the species level by *rpoB* and *gyrB* sequencing and multiplex-PCR-based analysis revealed that the isolate was *A. pittii*. Whole-genome sequencing of this *A. pittii* isolate determined the presence of bla_{OXA-96}, bla_{CARB-2}, and a novel $bla_{OXA-421}$ gene. The position of this novel $bla_{OXA-421}$ gene was similar to that of bla_{OXA-51} in *A. baumannii*, downstream of the phosphinothricin *N*-acetyltransferase gene and upstream of *fxsA* in the chromosome. This *A. pittii* isolate was found to belong to sequence type 119 (ST119). Here, we report the first isolation of IMP-4-producing *A. pittii* ST119 with a novel $bla_{OXA-421}$ gene from a patient in Australia and characterize its draft genome.

CASE REPORT

A 67-year-old diabetic woman suffered a fall leading to a displaced distal spiral tibial plateau fracture. In the weeks prior to the fall, she had received multiple antimicrobials (clindamycin, lincomycin, cephalexin, ciprofloxacin, and ceftazidime) for an infected hematoma of the breast and a series of lower respiratory tract infections. The patient underwent definitive repair of the fracture but postoperatively developed osteomyelitis. Debridement of the leg wound was performed. *Acinetobacter* species and vancomycin-resistant *Enterococcus* strains were isolated from the tissue removed. This *Acinetobacter* species (CR12-42) was carbapenem resistant. Despite ongoing antibiotic treatment, the patient's leg required amputation in March 2013, after continuous inflammation, infections for more than 5 months, and an episode of severe *Clostridium difficile* infection resulting in colectomy. The leg infection was resolved by the amputation.

The initial identification of this *Acinetobacter* species was done by Vitek 2. Antimicrobial susceptibility testing by Vitek 2 (bioMérieux) showed resistance to carbapenems, ceftazidime, ceftriaxone, cefepime, gentamicin, tobramycin, trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, and ciprofloxacin according to the EUCAST standard (1). The isolate was referred to our laboratory at the University of Queensland Centre for Clinical Research. The *Acinetobacter* isolate was identified to the species level by a *gyrB* multiplex PCR, which revealed that CR12-42 was *Acinetobacter pittii* (2). Partial *rpoB* sequencing (3) confirmed that CR12-42 was *A. pittii*.

Phenotypic characterization to determine the class of carbapenemase was performed as previously described (4–6). The *A. pittii* isolate showed a metallo-β-lactamase phenotype by producing a larger inhibition zone around carbapenem disks with EDTA than around carbapenem disks alone (>5-mm breakpoint increase in the size of the inhibition zone). The isolate also produced a positive result in the modified Hodge and Carba NP tests for carbapenemase production. MICs were determined with Etest (bioMérieux). The isolate was resistant to all of the carbapenems tested, i.e., ceftazidime, cefotaxime, cefepime, cefoxitin, ticarcillin-clavulanic acid, trimethoprim-sulfamethoxazole, and ciprofloxacin (Table 1). Interestingly, this *A. pittii* isolate was susceptible to tetracycline, minocycline, colistin, and tigecycline (Table 1).

Carbapenem resistance in *Acinetobacter* species is commonly associated with the presence of carbapenem-hydrolyzing class D β-lactamase- or oxacillinase-encoding genes such as bla_{OXA-23} and bla_{OXA-51} in *Acinetobacter baumannii* (7, 8). A PCR assay and sequencing for all of the bla_{OXA} genes frequently present in *Acinetobacter* species, i.e., $bla_{OXA-23-like}$, $bla_{OXA-51-like}$, $bla_{OXA-40-like}$, and $bla_{OXA-58-like}$, were performed (7–9). The isolate was positive for the $bla_{OXA-58-like}$ subclass and negative for other subclasses of bla_{OXA}. A PCR assay for IS*Aba1*, the common insertion element in *A. baumannii*, was also negative. A PCR assay and sequencing for other carbapenemase-encoding genes (10, 11), i.e., bla_{IMP}, bla_{NDM}, bla_{KPC}, and bla_{VIM}, were positive for bla_{IMP-4}. A prepared pair-ended library of the whole genomic DNA was sequenced via Illumina MiSeq to further characterize the resistance mechanisms of *A. pittii* CR12-42 and to analyze its genome.

Whole-genome DNA sequencing produced a total of 138,932,382 paired-end reads with 30× average coverage. We used the CLC genomic workbench version 7.5 (CLC Bio, Aarhus, Denmark) for *de novo* assembly with a 500-bp minimum threshold resulting in 127 contigs. The draft genome consisted of 4,372,178 nucleotides and was annotated by rapid annotations using subsystems technology (RAST) (12). RAST annotation showed that *Acinetobacter calcoaceticus* PHEA-2 (score, 503) and *Acinetobacter* sp. strain SH024 (score, 436) are the two closest neighbors of *A. pittii* CR12-42. Our isolate was related to only one other *A. pittii* strain, TG6411, but with a lower score of 221. A total of 13 *A. pittii* draft genomes have been described in the BioProject (http://www.ncbi.nlm.nih.gov/bioproject/); however, draft ge-

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TABLE 1 MICs of antimicrobials for *A. pittii* CR12-42 as determined by Etest

Antimicrobial(s)	MIC (mg/liter)	Interpretation[a]
Ertapenem	>32	Resistant
Imipenem	24	Resistant
Meropenem	12	Resistant
Doripenem	>32	Resistant
Cefepime	64	Resistant
Ceftazidime	>256	Resistant
Cefotaxime	>32	Resistant
Ceftriaxone	>32	Resistant
Cefuroxime	>256	Resistant
Cefoxitin	>256	Resistant
Piperacillin-tazobactam	12	Resistant
Ampicillin-sulbactam	2	Susceptible[b]
Ticarcillin-clavulanic acid	256	Resistant
Piperacillin	>256	Resistant
Amikacin	12	Intermediate
Gentamicin	>256	Resistant
Netilmicin	24	Resistant
Ciprofloxacin	3	Resistant
Tetracycline	0.75	Susceptible[b]
Minocycline	0.023	Susceptible[b]
Trimethoprim-sulfamethoxazole	>32	Resistant
Colistin	0.094	Susceptible
Tigecycline	0.094	Susceptible

[a] Unless noted otherwise, MIC interpretations are based on EUCAST criteria (1).
[b] Ampicillin-sulbactam, tetracycline, and minocycline MIC interpretations are based on CLSI criteria (33).

nomes of only three isolates were published, including one draft genome of an NDM-1-producing *A. pittii* strain from China (13).

In silico identification of CR12-42 to the species level by using *rpoB* and *gyrB* showed it to be 100% identical to *A. pittii*. *A. pittii* belongs, together with *Acinetobacter nosocomialis*, within the *A. calcoaceticus-baumannii* complex and was formerly named *Acinetobacter* genomic species 3 (14). *In silico* analysis of *A. baumannii* multilocus sequence typing (MLST) by the Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) identified *A. pittii* CR12-42 as being of sequence type 119 (ST119). The alleles found were *cpn-60* ($n = 36$), *fusA* ($n = 20$), *gltA* ($n = 38$), *pyrG* ($n = 16$), *recA* ($n = 38$), *rplB* ($n = 18$), and *rpoB* ($n = 20$). It has been reported that MLST by the Pasteur scheme is capable of providing the ST of *A. pittii* (15). The clinical significance of *A. pittii* ST119 is indicated by the fact that it has been reported to be the predominant clone among the *A. pittii* strains (18 out of 25) isolated in four hospitals in Japan (16). Interestingly, these Japanese *A. pittii* isolates possessed a different bla_{IMP} variant, bla_{IMP-19} (16). Of note, *A. pittii* ST119 has not been reported previously in Australia.

The resistance genes were screened with ResFinder (17). The β-lactamase-encoding genes bla_{IMP-4}, bla_{OXA-96}, and bla_{CARB-2} were identified. bla_{OXA-96} has a single nucleotide difference (a guanine-for-adenine substitution at position 483) from bla_{OXA-58}. bla_{OXA-96} had been reported within an *A. baumannii* isolate from Singapore that also harbored bla_{OXA-23} and bla_{OXA-64} (18). In our isolate, bla_{OXA-96} had a genetic context similar to that of bla_{OXA-58}, which was bracketed by IS*Aba3* (GenBank accession number JX968506) (Fig. 1).

In addition, a novel bla_{OXA} gene, $bla_{OXA-421}$, was identified (Fig. 1). This gene had a genetic environment identical to that of the chromosomal bla_{OXA-51} gene in *A. baumannii* (19), which includes two genes that are usually present upstream and downstream of bla_{OXA-51} in *A. baumannii*, the phosphinothricin *N*-acetyltransferase-encoding gene and *fxsA*, respectively. $bla_{OXA-421}$ has 95% identity with the previously reported bla_{OXA} gene (GenBank accession number CP002177, locus tag BDGL_000903) from the genome of *A. calcoaceticus* PHEA-2 (20), which is the closest neighbor of our CR12-42 isolate, as previously mentioned. The second closest relative of $bla_{OXA-421}$ was bla_{OXA} of *Acinetobacter oleivorans*, with 89% similarity (GenBank accession number CP002080, locus tag AOLE_1170) (21). The other bla_{OXA} genes similar to $bla_{OXA-421}$ were $bla_{OXA-324}$, $bla_{OXA-325}$, $bla_{OXA-326}$, $bla_{OXA-332}$, and $bla_{OXA-354}$ (88 to 89% similarity), which were recently identified in *A. calcoaceticus* (22). The carbapenemase activity of OXA-421 warrants further investigation.

The bla_{IMP-4} gene in *A. pittii* CR12-42 was located inside a class 1 integron. Downstream from bla_{IMP-4} were *qacG2* and the aminoglycoside and chloramphenicol resistance genes *aacA4* and *catB2* (Fig. 1). This genetic context of bla_{IMP-4} in CR12-42 was found to be identical to that in an IMP-4-producing *A. baumannii* strain from Singapore (GenBank accession number DQ532122) (18).

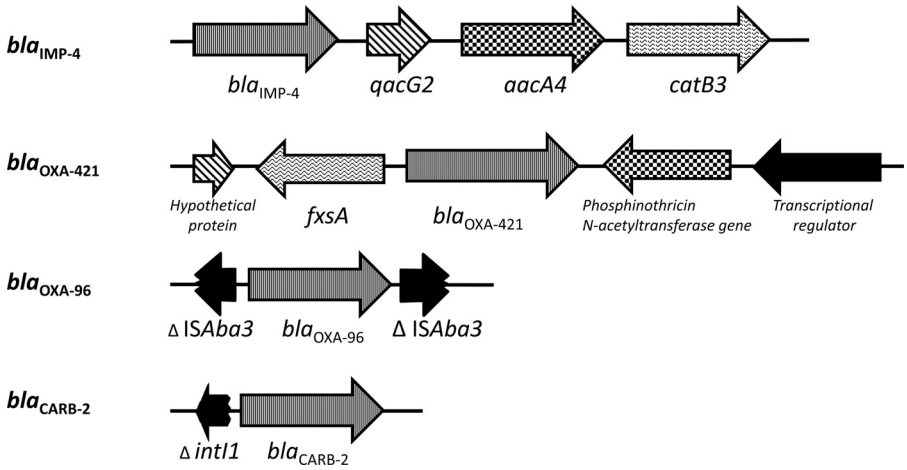

FIG 1 Genetic contexts of the four β-lactamase-encoding genes in *A. pittii* CR12-42.

bla_{IMP-4} has also been reported in *Acinetobacter junii* from Australia; however, the genetic context was not characterized (23). Our genetic context was also similar to that of bla_{IMP-4} in the IncHI2-type plasmid from *Enterobacter cloacae* and *Escherichia coli* and the IncL/M plasmid carrying bla_{IMP-4} in an *E. cloacae* strain from Australia (24, 25). However, the plasmid backbone of these sequences could not be identified within our draft genome. Further investigation is needed to determine if bla_{IMP-4} is located on a plasmid or the chromosome of CR12-42.

A carbenicillinase gene, bla_{CARB-2} was identified with ResFinder. bla_{CARB-2}, which was also designated bla_{PSE-1}, was first reported in *Pseudomonas aeruginosa* (26). The genetic context of bla_{CARB-2} in CR12-42 was also potentially a class 1 integron with a truncated integrase (*intI1*) located upstream of bla_{CARB-2} (Fig. 1). Other resistance genes found in this strain included *sul1* (sulfonamide resistance), *msr*(E) and *mph*(E) (macrolide resistance), and *aac-3-IId* (aminoglycoside resistance). Consistent with this, the *A. pittii* strain was resistant to gentamicin and tobramycin but susceptible to amikacin. Of note, no 16S rRNA methylase was found in this isolate.

Regardless of its resistance to multiple antimicrobials, *A. pittii* CR12-42 remained susceptible to tetracycline and minocycline, which was consistent with the absence of a tetracycline resistance gene within the draft genome. In addition, the MIC of ampicillin-sulbactam remained low (2 mg/liter), despite the presence of multiple carbapenemase-encoding genes. Further, sulbactam is known to have activity against *A. baumannii* (27). In a study by Higgins et al., the ampicillin-sulbactam MIC_{50} of 115 *A. baumannii* strains was 2 mg/liter (27). Ampicillin-sulbactam susceptibility was also shown in the majority of the previously reported *A. pittii* ST119 strains harboring bla_{IMP-19} (94%) in Japan (16). In addition, 94% of these were susceptible to minocycline, similar to the antimicrobial phenotype of CR12-42 (16). Apart from the difference in bla_{IMP} variants, CR12-42 has an antimicrobial phenotype and genotype identical to those of *A. pittii* ST119 from Japan.

IMP-producing *Enterobacteriaceae* strains have been frequently reported in Australia. Although OXA-23-like is the main subclass of carbapenemases identified in *A. baumannii*, IMP-4 is occasionally identified in *A. pittii* in locations such as Hong Kong and Singapore (18, 28). Other variants of bla_{IMP}, such as bla_{IMP-1}, bla_{IMP-8}, bla_{IMP-11}, and bla_{IMP-19}, have been described in *A. pittii* in Southeast Asia (16, 29, 30). *A. pittii* has also recently been reported to produce NDM (31, 32).

Generally, *A. baumannii* is considered the most important and the most prevalent *Acinetobacter* species causing infections. However, *A. pittii* has caused hospital outbreaks in The Netherland and China (32, 33) and was reported as the most common *Acinetobacter* species causing nosocomial infections in Germany (34). Our study illustrates the emergence of a multidrug-resistant *A. pittii* strain in Australia. Therefore, accurate identification to the species level and characterization of the prevalence of *A. pittii* among the *Acinetobacter* species isolated in our region and its antibiotic resistance warrant further investigation.

This work was approved by the Royal Brisbane and Women's Hospital Human Research Ethics Committee (HREC/13/QRBW/391: epidemiology, clinical significance, treatment, and outcome of infections by carbapenem-resistant *Enterobacteriaceae* and *Acinetobacter* species in Queensland). This project is registered as BioProject PRJNA255268 and BioSample SAMN03003652.

Nucleotide sequence accession numbers. The GenBank accession number of $bla_{OXA-421}$ is KM401566. The GenBank accession number of the draft genome of *A. pittii* CR12-42 is JQNT00000000.

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Chapter 3. Molecular epidemiology of carbapenem-resistant *A. baumannii* in a major hospital in Bangkok

3.1 Synopsis

Acinetobacter is an important and complex bacterial genus that causes a wide range of nosocomial infections [91]. A high prevalence of carbapenem-resistant *Acinetobacter baumannii* (CRAB) is observed worldwide including Asia [5, 6], which is a concerning threat in the healthcare system. Multiple outbreaks of CRAB have been reported from China, India, South Korea, Taiwan, Singapore and Thailand [86, 92-96].

This Chapter is a study of 300 isolates of *A. calcoaceticus – A. baumannii* complex from a 2200-bed tertiary care hospital situated in Bangkok, Thailand. We investigated molecular epidemiology and carbapenem resistance mechanisms in clinical isolates of *Acinetobacte*r spp. from Siriraj hospital, Bangkok, Thailand. In addition, 13 *Acinetobacter* spp. were selected for sequencing to study genomes of *Acinetobacter* spp. disseminated in this hospital. Through the genome analyses, a unique set of antimicrobial resistance and biofilm-related genes was identified. The presence and the stability of this particular set of these genes may contribute to its spread and persistence of *A. baumannii* IC2, which is the successful epidemic clone in this hospital

Predominance of international clone 2 OXA-23-producing *Acinetobacter baumannii* and insights into the genome of *Acinetobacter* spp. from Thailand

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SYNOPSIS

Objective: Carbapenem resistance in *Acinetobacter* spp. is an important problem in Thailand. We investigated the epidemiology and antimicrobial resistance genes of 300 non-repetitive clinical isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex obtained from Siriraj Hospital in Thailand.

Methods: The species identification was performed by partial *rpoB* gene sequencing. Isolates were subjected to PCR-based detection of antimicrobial resistance genes as well as allele-specific PCRs and repetitive sequence-based PCR (rep-PCR) for clonality assessments. The thirteen representative isolates were selected for full genome sequencing and analysis.

Results: *A. baumannii* was the main organism (*n*= 294, 98%) followed by *Acinetobacter nosocomialis* (*n*=4, 1.3%) and *Acinetobacter pittii* (*n*=2, 0.7%). The majority of *A. baumannii* isolates (*n*=236, 80%) belonged to international clone (IC) 2. Of 270 carbapenem-resistant *A. baumannii* (CRAB) isolates, all were resistant to ciprofloxacin, 92.2% were resistant to amikacin and 93% were resistant to cefepime. *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-40-like}}$ were found in 99.2% and 1.1% in CRAB isolates, respectively. The *armA* gene was present in 89% of aminoglycoside-resistant isolates. The *armA* gene was found within Tn*1548* and in close proximity to macrolide resistance genes (*mphE* and *msrE*) and *csu* locus which was responsible for biofilm formation. All IC2 isolates harboured an identical set of antimicrobial resistance genes; *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{ADC}}$, *strA*, *strB* and *tetB* and all biofilm-related genes.

Conclusion: The presence and the stability of this set of antimicrobial resistance and biofilm-related genes may contribute to its spread and persistence of *A. baumannii* IC2, which is the successful epidemic clone in this hospital.

INTRODUCTION

Acinetobacter is an important and complex bacterial genus that causes nosocomial infections, including ventilator-associated pneumonia, bacteraemia, wound infections, urinary tract infections and meningitis.[1] *Acinetobacter baumannii*, *Acinetobacter nosocomialis*, *Acinetobacter pittii* and *Acinetobacter calcoaceticus* are difficult to distinguish and usually misidentified. These four genomic species are grouped as "*A. calcoaceticus-A. baumannii* (ACB) complex". The former species are the most clinically important species whist *A. calcoaceticus* is rarely recovered from clinical specimens.[2] An emergence of carbapenem-resistant *A. baumannii* (CRAB) has caused a high burden for the healthcare system worldwide last decades. There are few drug options to treat CRAB infections, with the only options being tigecycline and colistin [1].

A high prevalence of carbapenem-resistant *Acinetobacter* spp. is observed in Asia.[3, 4] Multiple outbreaks of CRAB have been reported from China, India, South Korea, Taiwan, Singapore and Thailand.[5-10] *A. baumannii* was the primary causative pathogen for nosocomial pneumonia in tertiary care hospitals in Thailand and most of *A. baumannii* isolates were multidrug-resistant.[11] The prevalence rate of carbapenem-resistant *A. baumannii* isolates in Thailand was notably high. The percentage of *A. baumannii* which were carbapenem resistant was 2.1% in 2000 rising dramatically to 46.7% in 2005.[12] A multi-centre study in Thailand showed that the prevalence rate of carbapenem-non-susceptible *A. baumannii* isolates was 76.3% in 2010.[13]

Siriraj hospital is a 2300-bed tertiary care hospital situated in Bangkok, Thailand. *A. baumannii* was one of the main skin flora recovered from hospitalised patients at this hospital.[14] In 2012, the percentage of multidrug-resistant (including resistance to carbapenems) *A. baumannii* collected from hospitalised patients at Siriraj hospital was 88.7%.[15] Predominance and persistence of carbapenem-resistant *A. baumannii* in South East Asia including in Thailand has been reported.[16]

In this context of endemicity, we investigated the molecular epidemiology and carbapenem resistance mechanisms in clinical isolates of *Acinetobacte*r spp. from Siriraj Hospital, Bangkok, Thailand. In addition, 13 representative *Acinetobacter* spp. isolates were selected for full genome sequencing in order to provide a comparative genome analysis of the antimicrobial resistance mechanisms, resistance islands and biofilm formation on *Acinetobacter* spp. strains disseminated in this hospital.

MATERIALS AND METHODS

Bacterial isolates, antimicrobial susceptibility testing

A total of 270 non-repetitive clinical carbapenem-resistant *A. calcoaceticus-A. baumannii* (ACB) complex isolates were investigated. These isolates were collected from January to July 2010 from Siriraj Hospital - a 2200-bed University hospital in Bangkok, Thailand. The isolates were initially identified as ACB complex by using the Vitek2 automated identification system (BioMerieux, USA). Additionally, 30 isolates of carbapenem-susceptible ACB complex collected at the same period of time were also included in this study. The detail of the carbapenem susceptibility of the study isolates is illustrated in Table 1A and 1B.

Species identification was undertaken by using partial *rpoB* gene sequencing (zone 1) as previously described.[17] Antimicrobial susceptibility test by the disk diffusion method was performed to determine the susceptibility to amikacin, ciprofloxacin, cefepime, imipenem and meropenem according to the Clinical and Laboratory Standards Institute (CLSI) guideline.[18]

Genotypic characterisation of carbapenem resistance and resistance mediated by the 16S rRNA methyltransferase

Multiplex PCRs were performed on all isolates to characterise for oxacillinase genes ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA58-like}}$, $bla_{\text{OXA-143-like}}$ and $bla_{\text{OXA-235-like}}$)[19-21] and metallo-ß-lactamase genes (bla_{IMP}, bla_{VIM}, and bla_{NDM}) as previously described.[22-24] Additionally, a PCR for a region of IS*Aba1* upstream to $bla_{\text{OXA-51}}$ was carried out to detect the presence of this insertion element amongst *A. baumannii* isolates.[25]

The resistance to aminoglycoside due to the production of 16S rRNA methyltransferases (*armA*, *rmtB*, *rmtC* and *rmtD*) was also determined as previously described.[26] All primers used in this study were listed in Table S1.

Molecular typing

Allele-specific PCRs, designed to amplify *ompA*, *csuE* and $bla_{\text{OXA-51}}$, were used to determine sequence groups (SGs) in all *A. baumannii* isolates as previously described.[27, 28] Clonal analysis was performed on representative isolates by semi-automated rep-PCR DiversiLab™ (bioMerieux, VIC, Australia) according to the manufacturer's instruction. Isolates that clustered together with a similarity of 92% were considered to belong to the same rep-PCR types.

Whole genome sequence and analysis of Acinetobacter isolates

A total of 13 ACB isolates were subjected for sequencing to capture a range of genomic species, hospital locations, dates, genotypes and antimicrobial resistance phenotypes. The whole genome sequencing was performed using (HiSeq and Miseq) Illumina® paired-end technology (Australian Genome Research Facility and Diamantina Institute, Brisbane, Australia). The *de novo* assembly and sequence analyses were performed using CLC genomic workbench version 7.5 with the minimum 500 bp cut off. The *in silico* analyses of the genomes were performed by; (i) identification of antimicrobial resistance genes using ResFinder 2.1[29], (ii) *in silico* MLST (Pasteur schemes) using MLST 1.7[30], which are available on Centre for Genomic Epidemiology server (http://cge.cbs.dtu.dk/services/).

The genetic contexts of oxacillinase genes, *Acinetobacter* derived cephalosporinase (bla_{ADC}), 16S rRNA methyltransferase gene and other antimicrobial resistance genes were determined *in silico*. In addition, antimicrobial resistance mechanism caused by modification of target genes, such as mutations in the *gyrA* and *parC* and biofilm related genes were identified. The draft genomes were annotated using Rapid Annotations using Subsystems Technology (RAST).[31]

In *A. baumannii*, at least four genes, i.e. *bap* gene, *csu* locus, *bfmRS* and *pga* locus have been recognised to be associated with biofilm formation.[32, 33] Therefore, these four genes and/or operons were also determined and compared. The draft genomes were submitted to GenBank under BioProject PRJNA255268.The draft genome GenBank accession numbers are JRQT00000000, JPKX00000000, JRQS00000000, JRTX00000000, JRTY00000000, JRQU00000000, JRQV00000000, JRQX00000000, JRQY00000000, JRTZ00000000, JRQW00000000, JRQZ00000000 and JRUA00000000.

RESULTS

Identification of *Acinetobacter* spp. isolates

A total of 300 isolates previously identified as carbapenem-resistant members of ACB complex by Vitex2 identification system were further distinguished to species level using *gyrB* multiplex and the sequence of partial *rpoB* gene. Of the 300 clinical isolates, *A. baumannii*, *A. nosocomialis* and *A. pittii* were 294 (98%), 4 (1.3%), and 2 (0.7%), respectively. Most isolates were collected from sputum (*n*=232 or 77%), urine (*n*=23 or 7.7%), blood (*n*=16 or 5.3%) or wounds (*n*=12 or 4%) (Table 1A).

Of 270 carbapenem-resistant isolates, all were resistant to ciprofloxacin, 249 (92.2%) were resistant to amikacin and 252 (93%) were resistant to cefepime. In comparison, the resistance rate of 30

carbapenem-susceptible isolates to ciprofloxacin, amikacin and cefepime were 16.7%, 6.7% and 3.3%, respectively.

Resistance determinants of *Acinetobacter* spp.

The gene *bla*$_{\text{OXA-51-like}}$ was amplified (353 bp) in 291 (99%) *A. baumannii* isolates using previously described method.[21] The *bla*$_{\text{OXA-51-like}}$ was amplified with a larger product size (~1.5kb) in three isolates (T209, T222, T227). Of note, these three isolates had distinct colony morphology with pigment production on LB and MH medium. These isolates were confirmed as *A. baumannii* by *gyrB* amplification. The sequence of 1.5 kb of OXA-51-like PCR products showed that *bla*$_{\text{OXA-78}}$ (a variant of *bla*$_{\text{OXA-51}}$) was interrupted by IS*Aba19* in all three isolates.

All *A. baumannii* isolates were negative for IS*Aba1*, the common insertion element upstream to *bla*$_{\text{OXA-51-like}}$. Amongst carbapenem-resistant *A. baumannii* (*n*=269), 99.2% harboured *bla*$_{\text{OXA-23-like}}$. Three *A. baumannii* isolates harboured *bla*$_{\text{OXA-40-like}}$ with two isolates (T185 and T188) having both *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-40-like}}$ genes. The *bla*$_{\text{OXA-58-like}}$ gene was found in two *A. baumannii* isolates. However, these two isolates were phenotypically susceptible to carbapenems (Table 2).

One carbapenem-resistant *A. pittii* isolate harboured *bla*$_{\text{OXA-23-like}}$. All isolates were negative for *bla*$_{\text{OXA-143-like}}$, *bla*$_{\text{OXA-235-like}}$, and MBL genes tested (*bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{NDM}}$). 16S rRNA methyltransferase gene, *armA*, was present in 89% (223/250) of aminoglycoside-resistant isolates. Amongst carbapenem-resistant *A. baumannii* isolates (*n*=269), 248 isolates were also resistant to amikacin, broad spectrum aminoglycoside with 223 isolates harbouring *armA* (Table 2). None of the study isolates was positive for other 16s rRNA methyltransferase genes tested; *rmtB*, *rmtC* or *rmtD*.

Clonal analysis of the *Acinetobacter* spp.

Of the 294 *A. baumannii*, the majority of isolates (*n*=236, 80%) belonged to previously identified SG 1, which corresponds to international clone (IC) 2. Seventeen isolates belonged to SG 4. The remaining isolates belonged to SG 5 (*n*=15) or SG 7 (*n*=5). Twenty-one isolates gave an unrecognised pattern compared to previously described SGs and were diverse by DiversiLab^{TM}. All isolates of SG 1 and 4 were carbapenem-resistant *A. baumannii*.

Rep-PCR DiversiLab^{TM} was undertaken on 115 *A. baumannii*, four *A. nosocomialis* and two *A. pittii* isolates. The dendrogram results showed that 79 (65%) isolates were clustered in the largest group (>92% similarity), group A (Fig S1). The banding patterns of this cluster were similar to those identified as international clone 2 (IC2).[34] Interestingly, all group A isolates consisted of the isolates only from SG 1 and 4 and were carbapenem-resistant. Eight different groups and six singletons were

identified amongst carbapenem-susceptible *A. baumannii* isolates (*n*=25). Three isolates of *A. nosocomialis* were clustered together (>92% similarity) while two *A. pittii* isolates were not clonally related.

In silico analysis of *Acinetobacter* draft genome

The draft genome results and antimicrobial resistance genes of 13 *Acinetobacter* isolates which comprised of *A. baumannii* (*n*=11), *A. nosocomialis* (*n*=1) and *A. pittii* (*n*=1) are shown in Table 3. The size of the genome ranged from 3,680,364 to 4,362,838 bp. It is important to note that the smallest genome size was the *A. baumannii* with the fewest antimicrobial resistance genes. In contrast, *A. pittii* with the most number of antimicrobial resistance genes detected had the largest genome size. The difference of the genome size between these two strains was 682,474 bp. The results of *in silico* MLST of these isolates using Pasteur scheme was showed in Table 3. All *A. baumannii* rep-PCR group A isolates belonged ST2 except one isolate (T271) that belonged to ST215. *A. nosocomialis* and *A. pittii* isolates were ST279 and a novel ST655, respectively.

Carbapenem resistance mechanisms

In silico analysis of genome sequences of representative 11 *A. baumannii* isolates indicated that *bla*$_{\text{OXA-51}}$-like in these isolates were *bla*$_{\text{OXA-66}}$ (*n*=9), *bla*$_{\text{OXA-68}}$ (*n*=1) and *bla*$_{\text{OXA-120}}$ (*n*=1) (Table 3). The location of *bla*$_{\text{OXA-51-like}}$ was in between phosphinothricin N-acetyltransferase and *fxsA* genes, which is the usual chromosomal location of the *bla*$_{\text{OXA-51-like}}$ gene.[35] The *A. baumannii* with *bla*$_{\text{OXA-66}}$ were all ST2, and *bla*$_{\text{OXA-68}}$ and *bla*$_{\text{OXA-120}}$ belonged to ST215 and a novel ST653 (Table 3). IS*Aba1* was not detected adjacent to *bla*$_{\text{OXA-51-like}}$ in all isolates.

The genetic environments surrounding *bla*$_{\text{OXA-23}}$ in *A. baumannii and A. pittii* isolates were similar. The *bla*$_{\text{OXA-23}}$ in both species was bracketed by IS*Aba1* and was carried by Tn*2006* (GenBank accession no. JN129846).[36] The *bla*$_{\text{OXA-40-like}}$ in both *A. baumannii* isolates was *bla*$_{\text{OXA-72}}$ and was flanked by XerC/XerD recombination sites, which were presumed to be responsible for mobilisation of *bla*$_{\text{OXA-40-like}}$.[37-40]

Resistance island and other antimicrobial resistance mechanisms

The backbone of resistance AbaR4 was integrated into specific genomic site, *comM* (ATPase gene), which was identified in all isolates belonged to rep-PCR group A. AbaR4 is a common resistance island of international clone 2 isolates comprising the *uspA* (universal stress protein A), *sup* (sulphate permease), *tet(B)* (tetracycline resistance), *strB* (aminoglycoside resistance) and *strA* (aminoglycoside resistance) including Tn*2006* that carries *bla*$_{\text{OXA-23.}}$[36] The 16S rRNA

methyltransferase gene, *armA* in *A. baumannii* isolates was bracketed by transposase genes, *tnpU* and *tnpD* (Tn*1548*). The macrolide resistance genes, *mphE* and *msrE*, were found in close proximity to *armA* in all five *armA*-harboring isolates. Truncated IS*26* was also found downstream of *mphE* and *msrE* (Figure 1).

Acinetobacter derived cephalosporinase, bla_{ADC}, was present in all rep-PCR group A isolates. Two *A. baumannii* which showed carbapenem susceptibility lacked all the above antimicrobial resistance genes including bla_{ADC}. IS*Aba1* was detected upstream to chromosomal bla_{ADC} in all CRAB isolates. More than half of group A isolates harboured $bla_{\text{TEM-1}}$ (6/9, 67%). The carbapenem-resistant *A. pittii* isolate (T167) harboured several resistance genes including $bla_{\text{OXA-23}}$, $bla_{\text{OXA-10}}$, $bla_{\text{VEB-7}}$, *aadA1, aadB, aphA6, mphE, msrE, sul1, sul2, floR, cmlA1, aar-2* and *dfrA10.* (Table 3).

Sequences of quinolone resistance-determining region of the *gyrA* and *parC* genes revealed a change of Ser83 to Leu in *gyrA* and Ser80 to Leu in *parC* of almost all isolates. There was a Ser80 to Trp in the *parC* gene of *A. baumannii* T214.

***Biofilm-related genes in Acinetobacter* spp.**

Given the likely importance of biofilm formation to the success of *A. baumannii* in hospitals, biofilm formation associated genes, *bap*, *csu* locus, *bfmRS* and the *pga* locus, were identified (Table 4). The *bfmR* and *bfmS* genes and their promoter regions were present in all isolates. All four genes were detected in genomes of all *A. baumannii* ST2. The remaining isolates lacked either one or two of these genes (except *bfmRS*). The *csu* locus was located in close proximity with *armA* (in isolates harbouring *armA*).

DISCUSSION

A high prevalence of CRAB has been observed in Asia including Thailand.[13] In 2002, a study from Siriraj hospital showed that the percentage of *A. baumannii* collected from hospitalised patients which were MDR was more than 50% and this increased to 88.7% in 2012.[15] A subset of the total isolates collected at Siriraj hospital from January to July in 2010 was used in this study and included 270 carbapenem-resistant and 30 carbapenem-susceptible ACB complex. *A. baumannii* was the main organism (98%) followed by *A. nosocomialis* and *A. pittii* which is similar to reports from other countries.[41] Interestingly, all *A. nosocomialis* isolates was carbapenem-susceptible and recovered from sputum. Further, IS*Aba1* was not present in *A. nosocomialis* genome in the present study. This suggests that *A. nosocomialis* amongst our isolates may possess less ability to acquire antimicrobial

resistance determinants. *A. nosocomialis* also lacked several putative virulence related genes and had a lower mortality in clinical studies.[42, 43] However, a previous study showed that carbapenem resistance in *A. nosocomialis* was the consequence of an acquisition of *bla*$_{\text{OXA-23-like}}$ associated with IS*Aba1*.

Oxacillinase genes have been responsible for the carbapenem resistance mechanisms in *A. baumannii*. It has been reported that specific variants of OXA genes only present in certain geographical areas.[16] Similar to the worldwide situation of dominance of OXA-23 causing carbapenem resistance mechanisms in *A. baumannii*, *bla*$_{\text{OXA-23-like}}$ was also the main resistance determinant responsible for carbapenem resistance in *A. baumannii* in our hospital. Only three isolates of carbapenem-resistant *A. baumannii* harboured *bla*$_{\text{OXA-40-like}}$ were identified here. In contrast to the carbapenem-resistance phenotype observed amongst *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-40-like}}$-positive isolates, two *bla*$_{\text{OXA-58-like}}$-harbouring *A. baumannii* isolates remained susceptible to carbapenems (Table 2). It has been reported previously that *bla*$_{\text{OXA-58}}$-like-harbouring *Acinetobacter* spp. showed variable susceptibilities to carbapenems.[44] This might be caused by the lack of a strong promoter upstream of *bla*$_{\text{OXA-58-like}}$ in these isolates. Our study showed the first description of *bla*$_{\text{OXA-58}}$ from Thailand. A high prevalence of *Acinetobacter* spp. harbouring *bla*$_{\text{OXA-58}}$ was observed in Singapore, Taiwan and European countries.[16, 45-48]

The presence of *bla*$_{\text{OXA-51-like}}$ in all *A. baumannii* isolates emphasised that it is the naturally occurring gene found in *A. baumannii*. However, three *A. baumannii* isolates harbouring *bla*$_{\text{OXA-78}}$, a *bla*$_{\text{OXA-51-like}}$, were interrupted by IS*Aba19* resulting in a larger product size observed in multiplex PCR. This event was previously reported by Zander *et al*. in *A. baumannii* from Turkey and South Africa.[49] One of the limitations of this study, these three isolates were not whole genome sequenced, therefore the genetic context of the *bla*$_{\text{OXA-78}}$ cannot be analysed here. Thus, detection of intrinsic *bla*$_{\text{OXA-51}}$-like alone may not be accurate enough to identify *A. baumannii*. Additional methods such as *gyrB* multiplex PCR or sequencing methods based on the partial *rpoB* gene and the 16S-23S rRNA gene spacer region can be utilised for species identification.[17, 50-52]

bla$_{\text{VEB-type}}$ has been reported in *A. baumannii*.[53] However, there was only one report of *bla*$_{\text{VEB-3}}$ in *A. pittii* which was from Taiwan.[54] *A. pittii* T167 harboured *bla*$_{\text{VEB-7}}$ which was firstly recovered from *A. baumannii* (GenBank accession number FJ825622). The *bla*$_{\text{VEB-7}}$ in *A. pittii* T167 isolate was located in ~7kb class 1 integron structure containing *bla*$_{\text{VEB-7}}$-*addB-arr-2-cmlA-bla*$_{\text{OXA-10}}$-*aadA1* gene cassette array (Figure 2). This structure was a part of Tn*6061* originally identified in *Pseudomonas aeruginosa* and was also found in AbaR1, the largest resistance island (~86 Kb) in *A. baumannii* AYE strain.[55]

The coexisting rate of *armA* amongst all CRAB isolates and CRAB that belonged to IC2 (by rep-PCR pattern) was high, 82.8% and 87.3%, respectively. The genetic surrounding of *armA* was associated with Tn*1548* and similar to genomes of *A. baumannii* TYTH-1 (GenBank accession no. CP003856) and MDR-TJ (GenBank accession no. CP003500) isolated in Taiwan and China, respectively.[56, 57] This structure was also found in plasmids from *A. baumannii* MDR-ZJ06 (GenBank accession no. NC_017172)[58] and from many Enterobacteriaceae, such as pNDM-HK of *Escherichia coli* (accession no. HQ451074) from Hong Kong[59] (Figure 1).

A. baumannii isolates harbouring *armA* possessed identical set of antimicrobial resistance genes i.e. *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{ADC}}$, *bla*$_{\text{TEM-1}}$, *strA, strB, aphA1, mphE, msrE, sul2* and *tetB*. This set of antimicrobial resistance genes represented phenotypic characteristics of the majority of CRAB at Siriraj Hospital. In addition, the possession of four genes for biofilm formation were strongly associated with the ST2 regardless the slight variability of the antimicrobial resistance gene composition. Phenotypic of the biofilm formation of the isolates with different composition of biofilm forming genes requires further investigation.

Clonal spreads of CRAB have been reported previously in Thailand using different typing methods.[10, 60-62] Although SG typing showed that SG1 was the main sequence group of our isolates (80%), others SGs were also identified including SG4 (6%), SG5 (5%) and SG7 (2%). These SGs were also recovered from European countries; SG4 was found in the Netherlands, Turkey and Portugal, SG5 in Austria and Denmark and SG7 in Estonia.[27] Rep-PCR results showed that the majority of tested *A. baumannii* isolates (65%) belonged to IC2 and were resistant to carbapenem. The clonal spread of CRAB IC2 has also been reported from Asia-Pacific nations such as Australia, China, Japan and Singapore.[5, 63-65] In contrast, there was no predominant clone amongst carbapenem-susceptible *A. baumannii* isolates. This suggests that an acquisition of carbapenem resistance may be one of the adaptive attributes that cause a persistence of IC2. It is important to note that among the same rep-PCR group of our isolates included different STs or SGs. This suggested the drawbacks of semiautomated rep-PCR including limited discrimination of rep-PCR compared to MLST and the lack of manufacturer instructions on similarity cut-off values for classifying closely related rep-PCR profiles as identical genotypes.[66] However, MLST is primary useful for population genetics studies while rep-PCR has the advantage of being less time-consuming than MLST allowing for the investigation of large numbers of isolates.[34, 66]

Whole genome analysis of *A. baumannii* ST2 isolates (which clustered in IC2) indicated that all isolates harboured *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{ADC}}$, *strA*, *strB* and *tetB*. All genes associated with biofilm

formation were also present in ST2 isolates. Hence, the stability of these genes within the isolates belonging to IC2 may help the emergence and spread of this clone, which is the most successful epidemic clone in this hospital. The genome sequence of carbapenem-resistant *A. pittii* isolate showed that this isolate harboured different set of multiple antimicrobial resistance genes that were not detected amongst *A. baumannii* IC2 isolates. Class one integron structure was also detected, which facilitates further acquisition of antimicrobial, detergent and heavy metal resistance determinants in this *A. pittii* isolate. Continuous surveillance of *Acinetobacter* spp. and identification of their antimicrobial resistance mechanisms as well as screening scheme for colonisation amongst patients and environment in the hospital may be required for early detection and prevention of inter- and intra-species transfer of CRAB.

In conclusion, we demonstrated the homogenous spread of OXA-23-producing *A. baumannii* that belonged to IC2 at Siriraj hospital. Carbapenem-resistant *A. nosocomialis* and *A. pittii* was uncommon. The $bla_{\text{OXA-40-like}}$ and $bla_{\text{OXA-58-like}}$ carrying *A. baumannii* strains were also rare.

The genome sequences revealed the genotypic characteristics of carbapenem resistance and other multiple resistance as well as capability to form strong biofilm *A. baumannii* isolates within epidemic clone, IC2. Appropriate empirical antimicrobial treatment and infection prevention programs are pivotal to help preventing the further spread of this clone.

Tables and Figures

Table 1A. Specimen types

Specimen	Number of isolates						
	A. baumannii (294)		*A. nosocomialis* (4)		*A. pittii* (2)		Total (300)
	CS[a]	CR[b]	CS[a]	CR[b]	CS[a]	CR[b]	
BAL		1	-	-		-	1
Body fluid	-	5	-	-	-	-	5
Blood culture	-	15	-	-	1	-	16
CSF	-	1	-	-	-	-	1
Nasopharyngeal aspirate	-	1	-	-	-	-	1
Sputum	22	206	4	-	-	-	232
Tissue biopsy	-	5	-	-	-	-	5
Urine	2	20	-	-	-	1	23
Wounds (swab/pus)	1	11	-	-	-	-	12
Others	-	4	-	-	-	-	4

[a] CS; carbapenem-susceptible, [b] CR; carbapenem-resistant.

Table 1B. The location of patients when the specimens were collected.

Location	Number of isolates						
	A. baumannii (294)		*A. nosocomialis* (4)		*A. pittii* (2)		Total (300)
	CS[a]	CR[b]	CS[a]	CR[b]	CS[a]	CR[b]	
Medicine wards	2	127	1	-	1	1	132
ICUs	8	68	3	-	-	-	79
Surgery wards	7	17	-	-	-	-	24
OPD	3	18	-	-	-	-	21
Orthopaedic wards	-	8	-	-	-	-	8
Paediatric wards	1	3	-	-	-	-	4
Others	2	30	-	-	-	-	32

[a] CS; Carbapenem-susceptible, [b] CR; carbapenem-resistant.

Table 2. Oxacillinases and 16S rRNA methyltransferase genes detected by PCR in *Acinetobacter* spp.

Antimicrobial resistant genes	***A. baumannii* (*n*=294)**		***A. pittii* (*n*=2)**		***A. nosocomialis* (*n*=4)**
	Carbapenem susceptible (*n*=25)	Carbapenem resistance (*n*=269)	Carbapenem susceptible (*n*=1)	Carbapenem resistance (*n*=1)	Carbapenem susceptible (*n*=4)
bla$_{\text{OXA-51-like}}$	22 (88%) + 3[a]	267 (99.2%)	0	0	0
bla$_{\text{OXA-23-like}}$	0	267 (99.2%)	0	1	0
bla$_{\text{OXA-40-like}}$	0	3 (1.1%)	0	0	0
bla$_{\text{OXA-58-like}}$	2 (8%)	0	0	0	0
armA	0	223 (82.8%)	0	0	0

[a] Three isolates gave ~1.5kb product size.

Table 3. Selected genetic characteristics of *Acinetobacter* draft genomes

Isolate ID	Species	Specimen	Location	CR	SG	rep-PCR	MLST *in silico*	IS *Aba1*	Intrinsic *bla*$_{OXA}$	Acquired *bla*$_{OXA}$	16s rRNA methylase gene	Other resistance genes	Mutation in		Total bases (bp)
													gyrA	*parC*	
T7[a]	*A. baumannii*	CSF	ICU	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	*armA*	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aphA1, mphE, msrE, sul2, tetB*	S83L	S80L	3,938,556
T25[b]	*A. baumannii*	Wound swab	Surgery	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	*armA*	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aphA1, mphE, msrE, sul2, tetB*	S83L	S80L	3,862,654
T87[b]	*A. baumannii*	Blood culture	ICU	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	ArmA	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aphA1, mphE, msrE, sul2, tetB*	S83L	S80L	3,877,500
T122[a]	*A. baumannii*	Blood culture	Medicine	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	ArmA	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aphA1, mphE, msrE, sul2, tetB*	S83L	S80L	3,938,051
T173[a]	*A. baumannii*	Nasopharyngeal aspirate	Paediatric	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	ArmA	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aphA1, mphE, msrE, sul2, tetB*	S83L	S80L	3,936,842
T185[b]	*A. baumannii*	Blood culture	Surgery	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$, *bla*$_{OXA-72}$	-	*bla*$_{ADC}$, *strA, strB, aac(6')ii, sul2, tetB*	S83L	-	3,988,299
T188[b]	*A. baumannii*	Sputum	OPD	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$, *bla*$_{OXA-72}$	-	*bla*$_{ADC}$, *strA, strB, aac(6')ii, sul2, tetB*	S83L	-	3,984,620

T258[b]	*A. baumannii*	Sputum	Medicine	+	SG1	A	ST2	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	-	*bla*$_{ADC}$, *strA, strB, aac(6')ii, sul2, tetB*	S83L	S80L	3,950,019
T271[b]	*A. baumannii*	Urine	OPD	+	SG4	A	ST215	+	*bla*$_{OXA-66}$	*bla*$_{OXA-23}$	-	*bla*$_{ADC}$, *bla*$_{TEM-1}$, *strA, strB, aac(3')ic, aadA1, mphE, msrE,sul1, sul2, tetB*	S83L	S80L	3,868,725
T214[b]	*A. baumannii*	Sputum	ICU	-	SG5	E	ST10	-	*bla*$_{OXA-68}$	-	-	-	S83L	S80W	3,799,245
T229[b]	*A. baumannii*	Sputum	Paediatric	-	UN	I	ST653	-	*bla*$_{OXA-120}$	-	-	-	-	-	3,680,364
T167[b]	*A. pittii*	Urine	Medicine	+	NA	Single-ton	ST655	+	-	*bla*$_{OXA-10}$, *bla*$_{OXA-23}$		*bla*$_{VEB-7}$, *aadA1, aadB, aphA6, mphE, msrE, sul1, sul2, floR, cmlA1, aar-2, dfrA10*	S83L	S80L	4,362,838
T228	*A. nosocomialis*	Sputum	ICU	-	NA	M	ST279	-	-	-	-		-	-	3,797,192

[a] genome sequencing by using Ilumina MiSeq, [b] genome sequencing by Ilumina HiSeq., UN; unidentified, NA; not applicable.

Table 4. The presence of genes associated with biofilm formation identified by whole genome sequencing.

Isolate ID	Species	MLST[a]	*bap* gene	*csu* locus	*bfmRS*	*pga* locus
T7	*A. baumannii*	ST2	+	+	+	+
T25	*A. baumannii*	ST2	+	+	+	+
T87	*A. baumannii*	ST2	+	+	+	+
T122	*A. baumannii*	ST2	+	+	+	+
T173	*A. baumannii*	ST2	+	+	+	+
T185	*A. baumannii*	ST2	+	+	+	+
T188	*A. baumannii*	ST2	+	+	+	+
T258	*A. baumannii*	ST2	+	+	+	+
T271	*A. baumannii*	ST215	-	-	+	+
T214	*A. baumannii*	ST10	+	-	+	+
T229	*A. baumannii*	ST653	-	+	+	-
T167	*A. pittii*	ST655	-	-	+	+
T228	*A. nosocomialis*	ST279	+	+	+	+

[a]*In silico* MLST using Pasteur MLST scheme.

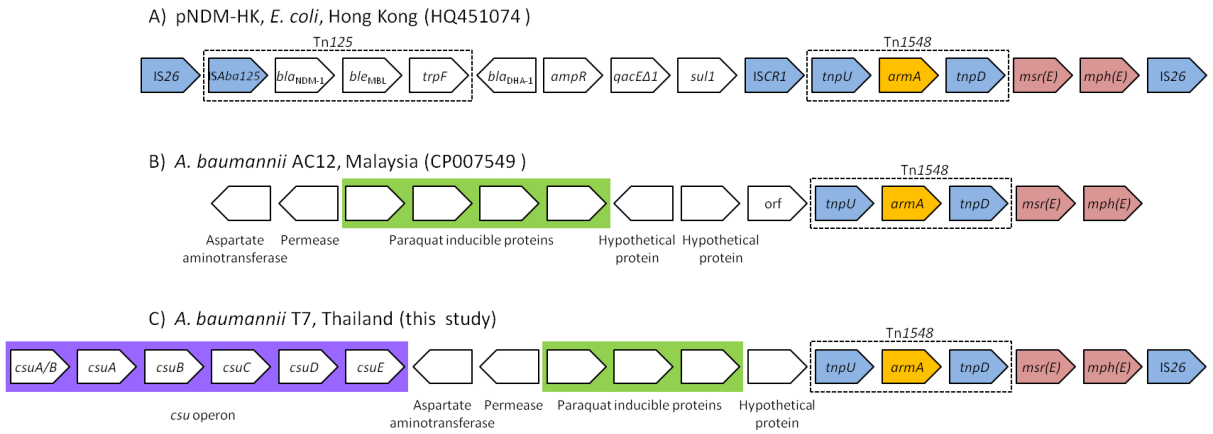

Figure 1. Genetic surrounding of Tn*1548* containing *armA*

Genetic surrounding of *armA* present in **A)** plasmid of *Escherichia coli* pNDM-HK from Hong Kong (HQ451074), **B)** *Acinetobacter baumannii* AC12 from Malaysia (CP007549) and **C)** *A. baumannii* T7 from Thailand in this study. Orientations of genes are indicated by arrows and the names are as given in or below the boxes. Yellow boxes represent *armA*, pink boxes represent macrolide resistance genes, blue boxes represent transposases/ IS elemments and white boxes represent others. Genes for *csu* operon are highlighted in purple and for paraquat inducible proteins are highlighted in green. The transposons are indicated within the dash line.

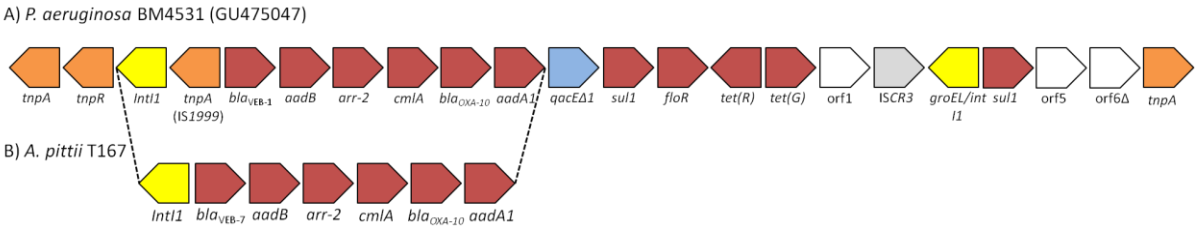

Figure 2. Genetic surrounding of *bla*VEB-7 in *A. pittii* isolate

A) Tn*6061* from *Pseudomonas aeruginosa* BM4531 (GU475047). **B)** The 7kb-contig containing antimicrobial resistance array in class 1 integron structure in *A. pittii* T167. Open arrows indicate coding sequences and direction of transcription. Colours are used to indicate ORF categories, red; antimicrobial resistance genes, orange; transposition module, yellow; integrase genes, grey; ISCR and white; other ORFs.

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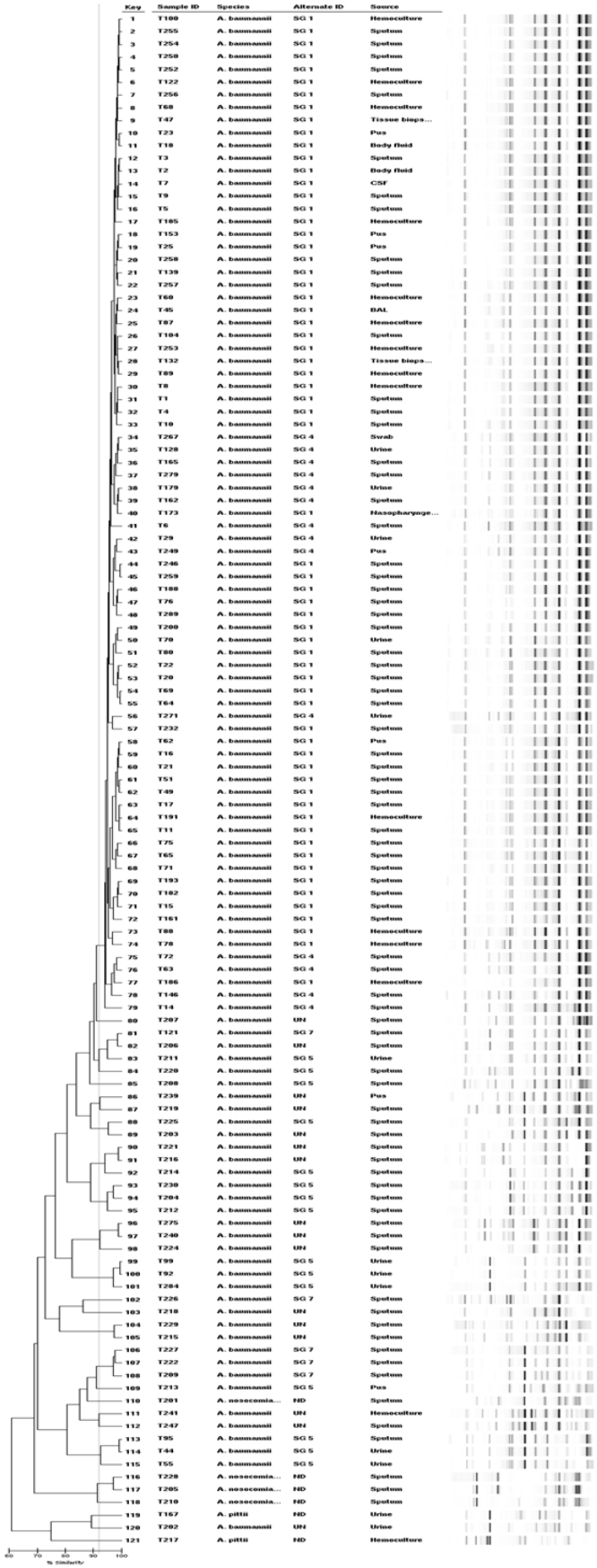
Supplementary data

Table S1. Primers used in this study

Primer	Target	Sequence (5' to 3')	Reference
Ac696F	*rpoB* Zone 1	TAYCGYAAAGAYTTGAAAGAAG	17
Ac1093R		CMACACCYTTGTTMCCRTGA	17
OXA-51_F	$bla_{\text{OXA-51-like}}$	TAATGCTTTGATCGGCCTTG	21
OXA-51_R		TGGATTGCACTTCATCTTGG	21
OXA-23_F	$bla_{\text{OXA-23-like}}$	GATCGGATTGGAGAACCAGA	21
OXA-23_R		ATTTCTGACCGCATTTCCAT	21
OXA-24_F	$bla_{\text{OXA-40/24-like}}$	GGTTAGTTGGCCCCCTTAAA	21
OXA-24_R		AGTTGAGCGAAAAGGGGATT	21
OXA-58_F	$bla_{\text{OXA-58-like}}$	AAGTATTGGGGCTTGTGCTG	21
OXA-58_F		CCCCTCTGCGCTCTACATAC	21
OXA-143-F	$bla_{\text{OXA-143-like}}$	TGGCACTTTCAGCAGTTCCT	20
OXA-143-R		TAATCTTGAGGGGGCCAACC	20
OXA-235_F	$bla_{\text{OXA-235-like}}$	TTGTTGCCTTTACTTAGTTGC	19
OXA-235_R		CAAAATTTTAAGACGGATCG	19
IMP-F	bla_{IMP}	CTACCGCAGCAGAGTCTTTGC	22
IMP-R		GAACAACCAGTTTTGCCTTACC	22
VIM-F	bla_{VIM}	GATGGTGTTTGGTCGCATA	23
VIM-R		CGAATGCGCAGCACCAG	23
NDM-F	bla_{NDM}	GCAGGTTGATCTCCTGCTTG	24
NDM-R		ACGGTTTGGCGATCTGGT	24
IS*Aba1*-F	upstream of $bla_{\text{OXA-51}}$	CACGAATGCAGAAGTTG	25
armA-F	*armA*	ATTCTGCCTATCCTAATTGG	26
armA-R		ACCTATACTTTATCGTCGTC	26
rmtB-F	*rmtB*	GCTTTCTGCGGGCGATGTAA	26
rmtB-R		ATGCAATGCCGCGCTCGTAT	26
rmtC-F	*rmtC*	CGAAGAAGTAACAGCCAAAG	26
rmtC-R		ATCCCAACATCTCTCCCACT	26
rmtD-F	*rmtD*	CGGCACGCGATTGGGAAGC	26
rmtD-R		CGGAAACGATGCGACGAT	26

Figure S1. rep-PCR (DiversiLab) dendrogram of 121 *Acinetobacter* spp. isolates and their specimen types, species and sequence groups. Grey line represents 92% cut off similarity.



Chapter 4. Genome of *A. baumannii*: a comparative analysis of isolates from four Asian countries

4.1 Synopsis

The clinical significance of carbapenem-resistant *A. baumannii* (CRAB) infections is widely recognised. However, there is limited understanding of the resistance to non-β-lactam antimicrobials and virulence of CRAB and carbapenem-susceptible *A. baumannii* (CSAB). Molecular approach has been implemented widely in the characterisation of *A. baumannii* in previous studies [29, 97-99]. However, the data are only available in countries with adequate research resources. To better understand the detail characteristics of the genome of *A. baumannii*, a total of 21 *Acinetobacter* spp. strains comprising 17 CRAB and 4 CSAB representative isolates from Thailand, Singapore, Malaysia and Japan were genome sequenced. Extensive molecular characterisation for carbapenem resistance mechanisms and molecular epidemiology had been performed on 340 study isolates prior to this isolate selection [100, 101].

In this study, the genome analysis has provided additional insights on the variability of the genomes of the successful clone of *A. baumannii* IC2 strains in particular the absence and presence of pathogenicity and resistance islands. In addition, the detailed genome analysis has provided insights of the capability of certain strains of *A. baumannii* to persist in the hospital environment. The potential challenges of the human humoral response in the infections by *A. baumannii* and in the development of vaccines are also evident. This clinical understanding was gained through genome analysis of the biofilm forming locus/operon and characterisation of lipooligosaccharides, respectively.

Insight into successful clone of *Acinetobacter baumannii* from Thailand, Japan, Malaysia and Singapore.

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ABSTRACT

Acinetobacter baumannii is an important cause of nosocomial infections due to its ability to acquire multiple antimicrobial resistance genes and to persist in hospital settings. The international clone (IC) 2 is a major clonal lineage of *A. baumannii* worldwide. We utilized the whole-genome sequencing approach to explore the diversity and genetic characteristics of 21 isolates of *A. baumannii* collected from major hospitals in Thailand, Singapore, Malaysia and Japan between 2009 and 2010. The majority of isolates were resistant to carbapenems and identified as IC2. The genetic contexts of intrinsic (*bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{ADC}}$) and important acquired-type resistance genes (*bla*$_{\text{OXA-23}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{TEM-1}}$ and *armA*) were elucidated.

AbaR4 resistance island was identified in the majority of the CRAB isolates (16 of 21 isolates) with the AbaR4-(I) as the dominant variant (12 of 16). Within this AbaR4, a Tn*2006* containing *bla*$_{\text{OXA-23}}$ that conferring carbapenem resistance was located. Resistance genes to non-β-lactam antimicrobials were also located inside the AbaR4-(I), i.e. *strA*, *strB*, *tetA*(B), *tetR*(B) and *sul2*. AbGRI2-1 which contained *bla*$_{\text{TEM-1}}$, *aacC1*, *aadA1* and *sul1* was identified in a non-IC2 isolate. AbaR3-type island was found in an IC1 isolate. Eleven isolates possessed *aphA1b*, an aminoglycoside resistance gene that was located in Tn*6020*. The resistance to broad spectrum aminoglycoside by *armA* was located within Tn*1548* and in close proximity with *mphE* and *msrE*, macrolide resistance. The class 1 integron containing *bla*$_{\text{IMP-4}}$ in one *A. baumannii* was identical to previously described in *A. pittii* and Enterobacteriaceae.

The comparative genome analysis revealed variations in the loci for the surface polysaccharide synthesis, K locus and OC locus, a core virulence factor. OCL1 was the dominant type of OC locus in IC2. The regions for type VI secretion system (T6SS) and initial adhesion and biofilm formation, *csu* operon in non-IC2 isolates were disrupted. AbaR4-(I) in combination with Tn*1548* had provided IC2 with resistance to nearly all antimicrobials available clinically. AbaR4-(I) resistance island, OCL1, T6SS and biofilm formation genes had contributed to the wide dissemination of *A. baumannii* IC2 within the Asia-Pacific region.

INTRODUCTION

Nosocomial infections caused by multidrug-resistant *Acinetobacter baumannii* have been recognised as a serious global problem [1]. The epidemic spread of international clonal lineages has been observed worldwide [2]. Amongst several clonal lineages, international clone (IC) 2 is the most predominant clone that is widely spread within hospitals in many countries, particularly in Asia-Pacific region where a high prevalence of antimicrobial resistance including resistance to carbapenems was already noted [2, 3]. The increase in carbapenem-resistant *A. baumannii* (CRAB) is mainly related to mobile genetic elements (such as transposons, insertion sequence (IS) elements and plasmids) which disseminate acquired antimicrobial resistance determinants [4]. In addition to the high propensity to develop and/or acquire resistance to multiple classes of antimicrobials, *A. baumannii* is known to be able to survive for a long period of time in hospital environment, which may lead to long-term persistence [5, 6].

In addition to the acquired antimicrobial resistance common in *A. baumannii*, genomic studies have demonstrated a high level of diversity within *A. baumannii* genomes even in the strains that are closely related. Frequently observed characteristics included variations in, (i) antimicrobial resistance islands (AbaR) such as AbaR4 (common in IC2), AbaR3 (common in ICI) and AbGRI, (ii) regions responsible for surface polysaccharide synthesis i.e. locus for capsule (K locus) and locus for outer core of lipid A moiety (OC locus), (iii) region for type VI secretion system (T6SS) and (iv) region for initial adhesion (*csu* operon). Our previous study indicated that genes associated with biofilm formation may be involved in the success of IC2 A. *baumannii* from Thailand [7]. These identified additional regions included *pga* locus and the *bap* gene.

Although the *A. baumannii* IC2 is acknowledged as a successful clonal lineage worldwide (particularly in Asia), the comprehensive genetic features related to hospital “adaptiveness” of this clone remain unclear. Our study aims to evaluate the genetic diversification and relationship amongst the genomes of IC2 isolates collected from different countries in Asia-Pacific and to determine their characteristics that may have contributed to the success of this clonal lineage within this geographical region by utilising a comparative genome approach.

MATERIALS AND METHODS

Bacterial isolates and their phenotypic and genotypic characterisation.

Acinetobacter spp. clinical isolates were collected during 2009-2010 from four tertiary-care hospitals in Thailand (*n*=300), Singapore (*n*=25), Malaysia (*n*=9) and Japan (*n*=6) from our previous studies [7, 8]. (Table S1). From this collection of isolates, 21 *A. baumannii* isolates were genome sequenced to capture a range of countries, antimicrobial resistance phenotypes, genotypes and clonal lineages (Table 2) comprising isolates form Thailand (*n*=11), Singapore (*n*=5), Japan (*n*=3) and Malaysia (*n*=2). These included 17 CRAB from all countries and four CSAB isolates from Thailand (*n*=2) and Japan *(n*=2).

Additional complete and draft *A. baumannii* genomes available on NCBI database were included in this study for genome content comparison and phylogenetic tree construction. These genomes and their GenBank accession number are ACICU (CP000863), AB0057 (CP001182), AYE (CU459141), ATCC17978 (CP000521), ATCC19606 (JMRY00000000) and MDR-ZJ06 (CP001937).

DNA preparation and whole-genome sequencing, assembly and annotation

Genomic DNA was extracted by using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories) according to manufacturer's instructions. Whole-genome sequencing was performed using Illumina HiSeq and MiSeq paired-end technology. Sequence data was assembled using CLC genomic workbench version 7.5 (CLC Bio, Aarhus, Denmark). Genome annotation was carried out by using an automated annotation system RAST (http://rast.nmpdr.org/) [9]

Genome analysis

MLST and antimicrobial resistance genes were determined via submission to the MLST finder 1.7 database [10] and ResFinder 2.1 database [11], respectively available from http://www.genomicepidemiology.org/. Contigs with the key carbapenemases and other β-lactamases were also manually annotated. The IS elements were identified using the IS Finder database (https://www-is.biotoul.fr/) [12]. The SNP analysis was performed through a web tool available at https://cge.cbs.dtu.dk/services/CSIPhylogeny/ to avoid the systematic biases caused by data generated by different platforms [13]. The phylogenetic trees were visualized and adjusted by using FigTree v1.4.2 available at http://tree.bio.ed.ac.uk/software/figtree/.

Nucleotide sequence accession numbers

The draft genomes obtained in this study were deposited in the GenBank database under BioProject PRJNA255268. The GenBank accession numbers are JPKX00000000, JRTX00000000 - JRTZ00000000, JRQS00000000 - JRQY00000000, LAIL00000000 - LAIO00000000, LAIY00000000 - LAIZ00000000 and LAKO00000000 - LAKR00000000

RESULTS

Phenotypic and genotypic characterisations

The 21 representative *A. baumannii* isolates comprised of 17 CRAB and 4 carbapenem-susceptible *A. baumannii* (CSAB) isolates. The antimicrobial susceptibility profiles of the 21 isolates were listed in Table 1. The 17 CRAB isolates were collected from Thailand (n=7), Singapore (n=4), Malaysia (n=2) and Japan (n=1). The four CSAB isolates were collected from Thailand (n=2) and Japan (n=2).

Our previous studies have indicated that the majority of the isolates from each country belonged to international clone 2 (IC2). The *in silico* MLST showed that most of the strains (*n*=15) belonged to sequence type 2 (ST2) using the Pasteur scheme [14] which corresponds to IC2 (Table 2). Of the ST2 isolates, 13 were CRAB while two were CSAB (both isolated in Japan). The core genome phylogenetic tree and SNP tree including our 21 strains and other six reference strains, where ACICU, strain of IC2, was utilised as a reference strain, revealed that most of the CRAB isolates were clustered together with the previously reported widely spread IC2 strain isolated from China MDR-ZJ06 (Figure 1) [15]. Two CSAB isolates from Japan were also a part of the IC2 cluster. One isolate from Singapore was grouped with IC1 strains, AYE from France and AB0057 from the United States (Figure 1).

Structure of antimicrobial resistance island

Several antimicrobial resistance islands were identified within our isolates. These included multiple variants of AbaR4-type, AbaR-3 derived island and recently described AbGRI2 [16-18].

AbaR4-type resistance islands in 16 isolates were identified truncating the ATPase gene *comM* while *comM* in carbapenem-susceptible isolates from Thailand remained intact (n=2). IC2 isolates (15) possessed three variants of AbaR4-type including the transposition module (*tni*), orf4 (hypothetical protein), *sup* (sulphate permease) and *uspA* (universal stress protein) as a backbone (Figure 2A). The AbaR4-(I) was the predominant type found in 12 isolates. This resistance island was also identified in a carbapenem-resistant isolate from Japan (J65) with IS*Aba17* inserted (Figure 2A), which was

designated as Tn6167 (GenBank accession number JN968483) in *A. baumannii* from Australia [18]. The AbaR4-(I) was 34.4 kb in length and carried many resistant genes, i.e. *strA*, *strB*, *tetA*(B), *tetR*(B), *sul2*. The remaining types of AbaR4-type islands lacked a 9.4-kb region containing the tyrosine recombinase gene *int* and seven open reading frames with unknown function.

sul2 (sulfonamide resistant dihydropteroate synthase) and *glmM* (glucosamine mutase) were not present in both AbaR4-(II) and AbaR4-(III). AbaR4-(III) also lacked a region containing transposition module adjacent to *int* region. AbaR4-(V) was found in one non-IC2 isolate from Malaysia. This AbaR variant did not contain the tetracycline resistance genes, *tetA*(B) and *tetR*(B) compared to other variants detected in IC2 isolates.

Another type of resistance island designated AbGRI2 (GenBank accession number JX869489) was also identified [17]. AbGRI2-1 was found in a non-IC2 isolate from Thailand. This 16.4 kb resistance island contained $bla_{\text{TEM-1}}$, a class 1 integron carrying the *aacC1*-orfP-orfP-orfQ-*aadA1* cassette array and *sul1* and multiple copies of IS*26*. The genetic content of this resistance island was similar to AbGRI2-1 with the absence of Tn*6020* containing *aphA1b*.

The class 1 integron region containing *aacA4-catB8-aadA1* cassette array and *sul1*, which was similar to the cassette array in AbGRI2-3 identified in the genome of MDR-ZJ06, was also detected in two isolates (S10 and J2770). However the location of the class 1 integron of these two isolates was different from MDR-ZJ06.

A CRAB isolate from Singapore, S36 was grouped together with reference genomes of IC1. The right side of AbaR3-type island, the original genomic island structure of *A. baumannii* of IC1 lineage [16], was detected the genome of S36. This consisted of a Tn*6019*-like transposase inserting and truncating *comM*, Tn*6018* and a part of multiple antimicrobial resistance region (MARR) containing *resX* (partial invertase/resolvase) region, *sul1*, the class 1 integron region carrying *aacC1*-orfP-orfQ-*aadA1* gene cassette array and *tnpA26* transposase of IS*26*. The sequence analysis showed that one copy of orfP was absent from this cassette array compared to that originally found in AbaR3. The left side of the AbaR3-type was unable to be identifed including the remaining part of the truncated *comM* in this isolate (Figure 2B).

Genetic context of acquired antimicrobial resistance genes

Regarding to carbapenem resistance, the $bla_{\text{OXA-23}}$ was found in all CRAB isolates expect one isolate that harboured $bla_{\text{IMP-4}}$. The $bla_{\text{OXA-23}}$ gene in all isolates was found in a transposon Tn*2006* composed of $bla_{\text{OXA-23}}$, APTase gene, DEAE helicase gene and *yeeA*, flanked by two copies of IS*Aba1*. In most of isolates, Tn*2006* was located in a genomic resistance island AbaR. The location for Tn*2006*

inserting into AbaR-4 was showed in Figure 2.Class A β-lactamase gene *bla*$_{TEM-1}$ was detected in nine isolates and was located in a transposon derived from Tn*1* containing a truncated *tnpR*. Another class A β-lactamase gene, *bla*$_{PER-1}$ was found in an isolate from Malaysia. The *bla*$_{PER-1}$ gene was associated with Tn*5393d* which also carried *aphA6b* conferring aminoglycoside resistance in this isolate. Another transposon known as Tn*6020* (flanked by two copies of IS26) was identified in eleven isolates carrying the aminoglycoside resistant gene, *aphA1b*.

The metallo-β-lactamase gene, *bla*$_{IMP-4}$ in S11 was located in a class 1 integron carrying the *bla*$_{IMP-4}$-qacG2-*aacA4*-*catB3* cassette array. The 16S rRNA methyltransferase gene, *armA* was located within Tn*1548* in ten isolates, flanked by transposase genes, *tnpU* and *tnpD*. The macrolide resistance conferring genes, *mphE* and *msrE*, were found in close proximity to *armA* in all *armA*-harboring isolates. Truncated IS*26* was also found downstream of *mphE* and *msrE*.

Intrinsic chromosomal resistance genes

Variations of the naturally occurring oxacillinase gene *bla*$_{OXA-51-like}$ were observed in different *A. baumanni*i isolates (Figure 3A). The *bla*$_{OXA-66}$ gene was found in all IC2 isolates (n=15) including T271 which did not belong to IC2 according to MLST and phylogenetic tree analyses. However, T271 was grouped together with IC2 isolates by rep-PCR, indicating that this may be related to IC2. The IC1 isolate possessed *bla*$_{OXA-69}$. *bla*$_{OXA-51}$, *bla*$_{OXA-65}$, *bla*$_{OXA-68}$ and *bla*$_{OXA-120}$ were identified amongst isolates that were neither IC1 nor IC2. In all *A. baumannii* isolates, *bla*$_{OXA-51-like}$ was not associated with IS*Aba1*; however, located between *fxsA* and phosphinothricin N-acetyltransferase-encoding genes.

The variants of *Acinetobacter*-derived cephalosporinase encoding gene *bla*$_{ADC}$ were identified in all isolates. The *bla*$_{ADC-30}$ gene was identified in five isolates and the sequence of 10 isolates showed 99% identity (1 SNP) to *bla*$_{ADC-30}$. One isolate contained *bla*$_{ADC-56}$. The remaining *bla*$_{ADC}$ sequences showed 99% similarity to *bla*$_{ADC-11}$ (n=2), *bla*$_{ADC-53}$ (n=1), *bla*$_{ADC-54}$ (n=1) and *bla*$_{ADC-64}$ (n=1). The presence of IS*Aba1* upstream of *bla*$_{ADC}$ was observered in most of isolates (n=16) which were all IC2 isolates and the IC2-related isolate, T271. The location of *bla*$_{ADC}$ was between GTP cyclohydrolase and putative outer membrane protein encoding genes.

Chromosomal gene losses: *Type VI secretion system (T6SS)*

The comparative genome analysis demonstrated that there were multiple events of chromosomal gene loss that may due to IS-mediated deletions (Figure 3). The large chromosomal region (49 kb) responsible for a type VI secretion system known as T6SS was absent in one carbapenem-susceptible isolate (T229) and one carbapenem-resistant isolate (M2). 29.5 kb of this region was absent in one carbapenem-resistance isolate form Singapore (S11), which possibly mediated by IS*Aba5* next to the deletion. The variation in T6SS region was observed in another susceptible isolate (T214) as well as an IC1 isolate (S36), while this region in all IC2 isolates was conserved.

Region for biofilm formation: csu operon

The regions of biofilm associated genes were investigated. The region harbouring genes responsible for chaperon-usher secretion system, CsuA/BABCDE known for pili assembly and biofilm formation was absent in two isolates. The presence of IS elements; IS*Aba1*, IS*Aba12* and IS*Aba125* adjacent to the *csu* operon observe in seven isolates including three isolate from Japan. However, in two isolates from Japan, insertion by IS element did not cause a deletion in the *csu* region. In The IS-mediated deletion (IS*26*) of entire region was also found in reference genome MDR-ZJ06. In the ACICU genome, IS*Aba125* was inserted between *csuA* and *csuA/B*. There was no variation in a two-component system BfmRS that regulated the *csu* operons amongst sequences of all isolates and reference strains. The gene encoding a complex biofilm-associated protein (Bap) was absent in three isolates (T229, T271 and S11) as well as in two reference strains, ATCC 17978 and ATTCC 19606. The *pga* locus encoding protein which synthesises surface polysaccharide and related to the strong biofilm phenotype was partially deleted by IS*Aba13* in one susceptible isolate T229.

Variation in the loci for surface polysaccharides synthesis

Variation in both OC and K loci was observed amongst genomes of 21 isolates. The OC locus is known for synthesis of the outer core of lipid A-core moiety and was previously explored [19, 20]. OCL1 type was the most predominant amongst these isolates. All IC2 isolates possessed the OCL1 while variation in this locus was identified in non-IC2 isolates; OCL1 (n=1), OCL5 (n=1), OCL6 (n=1) and OCL7 (n=1). In one carbapenem-susceptible isolate (T229), its OC locus was different from other previously reported loci. The genes encoding glycosyltransferase of new OCL shared the best blast matched with those in OCL7. However, the genes encoding nucleotide-sugar biosynthesis showed higher similarity to OCL6 (Figure 4). The structure of all OCL detected in this study was shown in Figure 4.

The locus for capsule biosynthesis, K locus, showed greater variation in comparison to OC loci across all isolates including isolates of IC2. The content and arrangement of the K locus, varied in different isolates. Four isolates have the KL3 which was identical to K locus in ATCC17978 with an IS*Aba1* inserting at the same position in two isolates. KL2 which previously identified in ACICU strain found in three isolates, of which one isolate had IS*Aba1* insertion. Two isolates possessed KL7 which was similar to K locus in *A. baumannii* TCDC-AB0715. One isolate belonged to IC1 had KL1 which was the K locus of AYE strain. The remaining variations of K locus were demonstrated in Figure 4.

DISCUSSION

Here we conducted whole-genome sequencing and a comparative genome analysis of 21 *A. baumannii* collected from different countries in Asia where IC2 is reported as a predominant clone. These strains included 17 CRAB and 4 CSAB from Thailand, Singapore, Japan and Malaysia with various genotypic and phenotypic characteristics. The majority of isolates belonged to IC2. Additionally, an isolate of IC1 (second common of clonal linage within this region), and isolates that not clonally related to IC2 or IC1 were also investigated. We sought a better understanding of the genomic diversity within IC2 and amongst other clonal lineages within the Asian region.

Antimicrobial resistance: islands and mechanisms

Resistance island

AbaR4-type resistance islands are known to be characteristic of the IC2 isolate. Here, we identified 6 variants of AbaR4 and found that AbaR4-(I) was the predominant variant in IC2 isolates across all countries. AbaR4-(III) was the only variant observed in the non-IC2 strain from Malaysia. This AbaR-4-(III) type lacked a region containing tetracycline resistance genes which correlates with the observed tetracycline susceptible phenotype. The acquisition of Tn*2006* carrying *bla*$_{\text{OXA-23}}$, was the main mechanism observed conferring carbapenem resistance. Tn*2006* is frequently found in AbaR-4 of IC2 isolates; however we identified Tn*2006* in our non-IC1 isolates. Additionally, two IC2 isolates from Japan harboured the AbaR4-(I) island without Tn*2006*. Tn*2007* and Tn*2008* which had been reported in USA and Europe were not found amongst our study isolates [21]. This suggests that AbaR4-(I) variant regardless the presence of Tn*2006* was the characteristics of *A. baumannii* IC2 in this region.

ISAba1: association with intrinsic β-lactamases

The recombination and mutation events were usually identified in the intrinsic *bla*$_{\text{OXA51-like}}$ and *bla*$_{\text{ADC}}$ genes [22]. IS element IS*Aba1* was also recognised acting as a promoter region associated with the expression of these genes [23, 24]. However, the *bla*$_{\text{OXA-51-like}}$ gene in all IC2 isolates was *bla*$_{\text{OXA-66}}$ without the presence of IS*Aba1* upstream. This may suggest that all carbapenem resistance in our IC2 isolates was related to acquired-type OXAs while OXA-66 alone is not enough to confer carbapenem resistance, observed in two carbapenem-susceptible IC2 isolates harbouring *bla*$_{\text{OXA-66}}$ from Japan. Most of *bla*$_{\text{ADC}}$ amongst IC2 isolates was *bla*$_{\text{ADC-30}}$ and its variant. IS*Aba1* was found upstream of *bla*$_{\text{ADC}}$ variants in IC2 isolates while other variants, *bla*$_{\text{ADC-11}}$, *bla*$_{\text{ADC-53}}$, *bla*$_{\text{ADC-54}}$ and *bla*$_{\text{ADC-64}}$ of non-IC2 isolates were not associated with IS*Aba1*. The cephalosporin resistance phenotypes in studied isolates were correlated to the presence of IS*Aba1* upstream of *bla*$_{\text{ADC}}$.

Other resistance mechanisms

Regarding the antimicrobial susceptibility of ampicillin-sulbactam, the correlation between the presence of *bla*$_{\text{TEM-1}}$ and ampicillin-sulbactam resistance phenotype was observed. Our findings supported the notion that TEM-1 may represent a clinically relevant mechanism of sulbactam resistance in *A. baumannii* [25]. Even though the metallo-β-lactamase encoding gene, *bla*$_{\text{IMP-4}}$ was found in only one isolate from Singapore, the order of gene cassette array carried by class 1 integron (*bla*$_{\text{IMP-4}}$-qacG-*aacA4-catB3*) was identical to those previously identified in various Enterobacteriaceae and *Acinetobacter* spp. in Singapore, China, the Philippines and Australia [26, 27]. The frequent presence of this resistance gene array in a broad host range may suggest the importance of *bla*$_{\text{IMP-4}}$ array for carbapenem resistance in the Asia-Pacific regions.

Variations in regions for pathogenicity and persistence

The regions responsible for surface polysaccharide synthesis showed variation across all isolates and within IC2 isolates. The K locus amongst IC isolates was highly variable with 7 variations detected. Interestingly, four isolates of IC2 had a similar K locus to *A. baumannii* ATCC17978 strain that was not a strain of IC2. On the contrary, the OC locus is less variable with the predominant type OCL1 identified in all IC2 isolates and IC2 reference strains, ACICU and MDR-ZJ06. It is known that surface polysaccharide is highly immunogenic and is an important virulence factor. Thus, the interaction with host immune system affecting this region may explain a unique form of OC locus observed amongst our IC2 isolates. Moreover, such variations observed of the K locus may impact in the difficulties to generate human immune response to *A. baumannii*.

Another conserved region in IC2 isolates is the T6SS region, present in all IC2 isolates. The entire region was absent in three non-IC2 isolates which may cause by IS-mediated deletion. The role of T6SS in *A. baumannii* is possibly involved in inter-bacterial interactions and competition with other bacteria species [28, 29]. The homology observed in this region amongst IC2 isolates may imply that this region is pivotal for *A. baumannii* IC2 to predominate and persist in environment.

A similar incidence was observed in the adherence and biofilm associated region, *csu* region. The *csu* region was present in most of IC2 isolates while complete or partial deletions of this region were detected in six non-IC2 isolates. This suggests that the *csu* region might responsible for *A. baumannii* persistence, particularly for the IC2 clonal lineage. The minor variations in *csu* operon were found in three IC2 isolates collected from Japan. This may explain the different situation in Japan, where the prevalence of carbapenem-resistant *A. baumannii* is still low in spite of the heavy use of carbapenems [30]. However, only three isolates collected from Japan were investigated in this study. Further investigation and more studied isolates may be required for additional understanding of *A. baumannii* IC2 in Japan.

In summary, despite a high genomic diversity amongst *A. baumannii* genomes, the unique genetic contexts of our IC2 Asian isolates were observed in the present study comprising (i) intrinsic β-lactamase genes; $bla_{\text{OXA-66}}$, and $bla_{\text{ADC-30-like}}$ associated with IS*Aba1* upstream, (ii) resistance island AbaR4-(I), (iii) the OC locus type OCL1 and (iv) intact TS66 and *csu* operon regions. Although our approach is a snapshot paradigm to explore population and genetic structure of *A. baumannii* from different geographic locations, we have identified the areas of interest which may need further investigation to elucidate factors leading to the success of this clone.

CONCLUSION

To our knowledge, this is the first comparative genomic study to include isolates from multiple countries from Asia. The analysis of 21 *A. baumannii* genome sequences revealed a large diversity amongst our isolates. In addition to variable antimicrobial resistance genes and mobile elements, we identified characteristics found in IC2 isolates including the locus involved in surface polysaccharide synthesis and the chromosomal regions associated with bacterial interactions and biofilm formation. Taken together, these characteristics in combination with antimicrobial resistance genes may have contributed to the success and persistence of this international clone in the Asia-Pacific region.

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Table 1. Disk susceptibility testing of *Acinetobacter* spp.

Isolate ID	Country of origin	CRAB	Antimicrobial susceptibility by disc diffusion testing[1]											
			MEM	IPM	CTX	CAZ	FEP	SAM	TZP	TIM	AM	CIP	TE	SXT
T7	Thailand	+	R	R	R	R	R	R	R	R	R	R	R	R
T25	Thailand	+	R	R	R	R	R	R	R	R	R	R	R	R
T87	Thailand	+	R	R	R	R	R	R	R	R	R	R	R	R
T122	Thailand	+	R	R	R	R	R	R	R	R	R	R	R	R
T173	Thailand	+	R	R	R	R	R	R	R	R	R	R	R	R
T185	Thailand	+	R	R	R	R	R	S	R	R	R	R	R	R
T188	Thailand	+	R	R	R	R	R	S	R	R	R	R	R	R
T214	Thailand	-	S	S	I	S	S	S	S	I	S	R	S	S
T229	Thailand	-	S	S	S	S	S	S	S	S	S	S	S	S
T258	Thailand	+	R	R	R	R	R	S	R	R	R	R	R	R
T271	Thailand	+	R	R	R	R	I	I	R	R	S	R	R	R
J65	Japan	+	R	R	R	R	R	S	R	R	S	R	I	R
J133	Japan	-	S	S	R	R	R	S	S	S	S	R	R	R
J2770	Japan	-	S	S	R	R	S	S	I	S	R	R	R	S
M1	Malaysia	+	R	R	R	R	R	R	R	R	R	R	R	R
M2	Malaysia	+	R	R	R	R	R	R	R	R	R	R	I	R
S10	Singapore	+	R	R	R	R	R	R	R	R	R	R	R	R
S11	Singapore	+	R	R	R	R	R	S	S	R	S	R	S	I
S19	Singapore	+	R	R	R	R	R	S	R	R	R	R	R	R

S36	Singapore	+	R	R	R	R	R	S	R	R	R	R	S	I
S46	Singapore	+	R	R	R	R	R	I	R	R	R	R	R	R

Note: CRAB = carbapenem-resistant *Acinetobacter baumannii*, [1]Interpreted using CLSI 2014, MEM = meropenem, IPM = imipenem, CTX = cefotaxime, CAZ = ceftazidime, FEP = cefepime, SAM = ampicillin-sulbactam, TZP = piperacillin-tazobactam, TIM = ticarcillin-clavulatnate, AM = amikacin, CIP = ciprofloxacin, TE = tetracycline, SXT = trimethoprim-sulphamethoxazole.

Table 2. Selected phenotypic and genetic characteristics of *Acinetobacter baumannii* draft genomes

Isolate ID	Country of origin	Specimen	CRAB	rep-PCR	MLST *in silico*		β-lactamases					16s rRNA methylase	Other resistance genes	Mutation in	
					Pasteur	PubMLST	Intrinsic OXA	Acquired OXA	MBL	ADC	Class A			*gyrA*	*parC*
T7	Thailand	CSF	+	A	ST2	ST208	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB, aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
T25	Thailand	Wound swab	+	A	ST2	ST195	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB, aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
T87	Thailand	Blood culture	+	A	ST2	ST457	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB, aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
T122	Thailand	Blood culture	+	A	ST2	ST436	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB, aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
T173	Thailand	Nasopharyngeal aspirate	+	A	ST2	ST195	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB, aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
T185	Thailand	Blood culture	+	A	ST2	NEW-1	OXA-66	OXA-23, OXA-72	-	ADC-30*	-	-	*strA, strB, aac(6')ii, sul2, tet*(B)	S83L	-
T188	Thailand	Sputum	+	A	ST2	NEW-1	OXA-66	OXA-23, OXA-72	-	ADC-30*	-	-	*strA, strB, aac(6')ii, sul2, tet*(B)	S83L	-
T214	Thailand	Sputum	+	A	ST2	ST368	OXA-66	OXA-23	-	ADC-30*	-	-	*strA, strB, aac(6')ii, sul2, tet*(B)	S83L	S80L

T229	Thailand	Urine	+	A	ST653	NEW-2	OXA-66	OXA-23	-	ADC-30*	TEM-1	-	*strA, strB, aacC1, aadA1, mphE, msrE,sul1, sul2, tet*(B)	S83L	S80L
T258	Thailand	Sputum	-	E	ST10	ST585	OXA-68	-	-	ADC-53	-	-	-	S83L	S80W
T271	Thailand	Sputum	-	F	ST215	NEW-3	OXA-120	-	-	ADC-64	-	-	-	-	-
J65	Japan	Clinical	+	A	ST2	ST208	OXA-66	OXA-23	-	ADC-30	-	-	*strA, strB, sul2, tet*(B)	S83L	S80L
J133	Japan	Blood culture	-	A	ST2	ST208	OXA-66	-	-	ADC-56	-	-	*strA, strB, sul2, tet*(B)	S83L	S80L
J2770	Japan	Clinical	-	A	ST2	ST473	OXA-66	-	-	ADC-30	-	ArmA	*strA, strB,aphA1b, aacA4, aadA1, mphE, msrE, sul1, tet*(B), *catB8*	S83L	S80L
M1	Malaysia	Clinical	+	A	ST2	ST195	OXA-66	OXA-23	-	ADC-30*	TEM-1	ArmA	*strA, strB,aphA1b, mphE, msrE, sul2, tet*(B)	S83L	S80L
M2	Malaysia	Clinical	+	C	ST16	ST355	OXA-51	OXA-23	-	ADC-11*	PER-1	-	*strA, strB,aphA6, aphB6, mphE, msrE, sul2, tet39*	S83L	S80L
S10	Singapore	Clinical	+	A	ST2	NEW-4	OXA-66	OXA-23	-	ADC-30	TEM-1	ArmA	*strA, strB,aphA1b, aacA1, aadA1, mphE, msrE, sul1, tet*(B), *catB8*	S83L	S80L
S11	Singapore	Clinical	+	D	ST654	NEW-5	OXA-65	-	IMP-4	ADC-54*	-	-	*aphA1b, aphA6, aacA4, aac(3')-iid, mphE, msrE, sul1, catB3*	-	-
S19	Singapore	Clinical	+	A	ST2	ST218	OXA-66	OXA-23	-	ADC-30	-	ArmA	*strA, strB,aphA1b, aacA4, aadA1, mphE, msrE, sul1, tetB, catB8,*	S83L	S80L

S36	Singapore	Clinical	+	B	ST1	ST491	OXA-69	OXA-23	-	ADC-11*	-	-	*aphA6, aacC1, aadA1, sul1*	S83L	S80L
S46	Singapore	Clinical	+	A	ST2	ST219	OXA-66	OXA-23	-	ADC-30	-	ArmA	*strA, strB,aphA1b, accA4, aadA1, sul1, tet*(B), *catB8*	S83L	S80L

*99% similarity variant

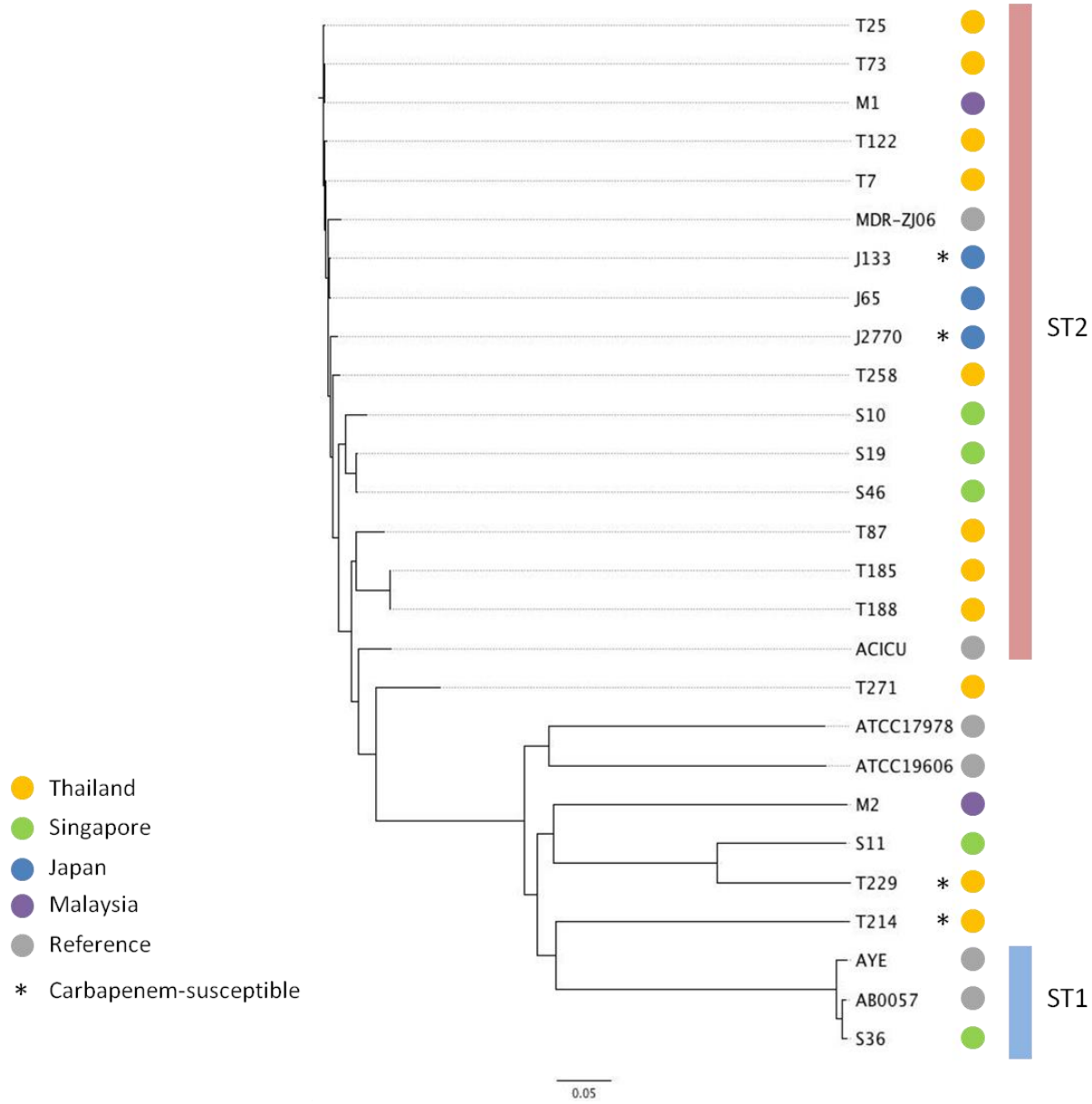

Figure 1. Phylogenetic tree of 21 *A. baumannii* isolates and 6 reference strains.

The phylogenetic tree was constructed based on the basis of SNPs and was rooted with *A. baumannii* ACICU. Circles were colour coded by the country of origin with reference genomes coded in grey, where yellow indicates Thailand, blue indicates Japan, purple indicates Malaysia and red indicates Singapore. * represents carbapenem-susceptible isolate.

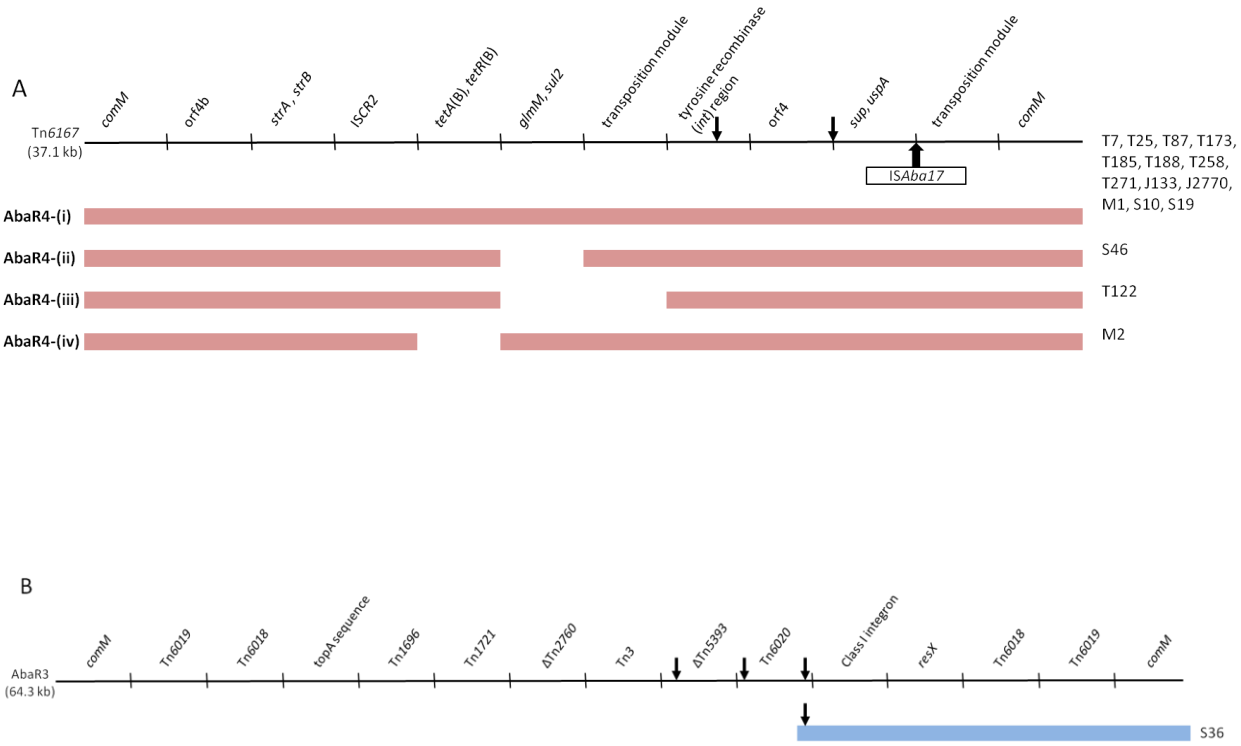

Figure 2. Structure of resistance island found in this study.

The colour bars represent region that was homologous to the backbone. A) AbaR4-type variants. The downward arrows represent Tn*2006* insertion sites. The Upward arrow represents the position of IS*Aba17* interruption in Tn*6167*. B) AbaR3-type island. Arrows indicate the position of IS*26* presented.

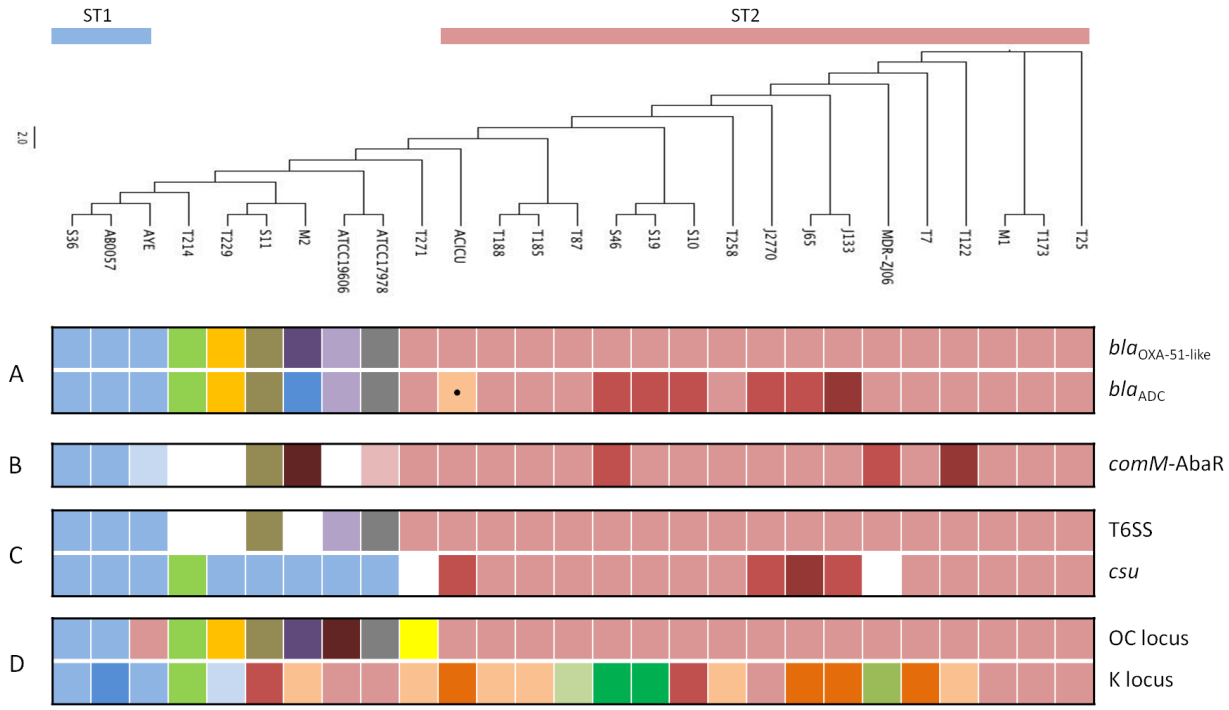

Figure 3. Distribution of variations in each region from comparative genome analysis.

The phylogenetic tree from Figure 1 was placed at the top to correlate isolates with their genome features. Blue and pink shades generally represent the most common type of variation in IC2 and IC1 respectively. Different colours indicate other variations unique to an individual isolate. Blank indicates that a region was not present. **A)** Intrinsic β-lactamase alleles. Pink colors represent *bla*$_{\text{OXA-66}}$ for *bla*$_{\text{OXA-51-like}}$ allele and *bla*$_{\text{ADC-30-like}}$ in *bla*$_{\text{ADC}}$ allele. Black dot indicates frameshift mutation of *bla*$_{\text{ADC}}$ in ACICU. **B)** AbaR type interrupted *comM*. Pink shades represent AbaR-4 type with the dominant AbaR4-(I). Blue shades represent AbaR3-type. **C)** Chromosomal region loss. Pink color represents the presence of T6SS and *csu* region. Other colors represent different types of variants unique to the isolate. **D)** Surface polysaccharide. For OC locus, pink color represents OCL1, blue represents OCL3. In K locus, pink shades represent KL2 and KL2-like.

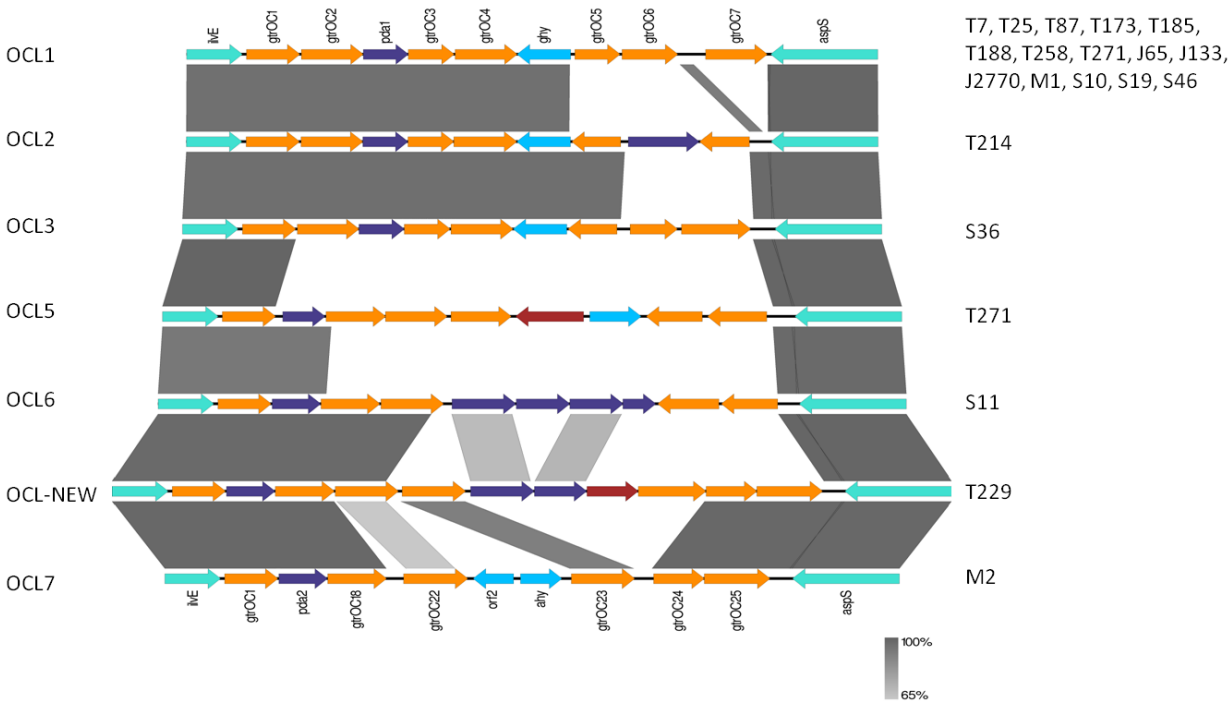

Figure 4. A schematic representation of all OC locus variants identified within this study.

The linear comparison figure of OC loci was generated by using Easyfig 2.1 available from http://easyfig.sourceforge.net/. Arrows represent genes and their direction of transcription. The color scheme represents the predicted function of gene products – cyan; flanking, orange; glycosyltransferase (*gtrOC*), purple; nucleotide-sugar synthesis, red; acyl-transferase and blue; others.

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Table S1. Genome sequence information used in 21 *Acinetobacter baumannii* isolates

Isolate ID	Country of origin	Source	Illumina	Total length	No. of Contigs	N_{50}	Average contig length	%GC content	No. of CDs	No. of RNAs	Accession no.
T7	Thailand	CSF	MiSeq	3,938,552	71	113,955	55,473	38.8	3,742	66	JRQT00000000
T25	Thailand	Wound	HiSeq	3,862,654	80	141,246	48,283	38.9	3,648	66	JPKX00000000
T87	Thailand	Blood culture	HiSeq	3,877,500	77	148,892	50,357	38.9	3,689	67	JRQS00000000
T122	Thailand	Blood culture	MiSeq	3,938,051	83	109,995	47,446	38.9	3,772	61	JRTX00000000
T173	Thailand	Nasopharygeal aspirate	MiSeq	3,936,792	68	119,860	57,894	39	3,744	63	JRTY00000000
T185	Thailand	Blood culture	HiSeq	3,987,745	102	141,629	39,096	38.9	3,843	67	JRQU00000000
T188	Thailand	Sputum	HiSeq	3,985,383	108	130,770	36,895	38.9	3,806	66	JRQV00000000
T258	Thailand	Sputum	HiSeq	3,799,157	38	205,362	99,978	38.9	3,577	66	JRTZ00000000
T271	Thailand	Sputum	HiSeq	3,679,512	125	74,692	29,436	39	3,507	67	JRQW0000000
T214	Thailand	Sputum	HiSeq	3,949,699	102	96,802	38,723	39.1	3,787	67	JRQX00000000
T229	Thailand	Urine	HiSeq	3,868,725	89	105,643	43,469	39	3,658	66	JRQY00000000
J65	Japan	Unknown	HiSeq	3,897,454	62	113,238	62,862	39	3,699	68	LAKO00000000
J133	Japan	Blood culture	MiSeq	3,887,746	114	102,009	34,103	39	3,696	65	LAKQ00000000
J2770	Japan	Unknown	MiSeq	3,944,409	103	102,056	38,295	39	3,736	63	LAKR00000000
M1	Malaysia	Unknown	MiSeq	3,869,793	89	106,127	43,481	39	3,651	65	LAIL00000000
M2	Malaysia	Unknown	HiSeq	4,132,915	122	101,807	33,876	38.9	3,984	69	LAKP00000000

S10	Singapore	Unknown	MiSeq	4,073,148	88	110,786	46,286	38.8	3,895	67	LAIM00000000
S11	Singapore	Unknown	HiSeq	4,125,959	206	47,424	20,029	39.1	4,026	68	LAIY00000000
S19	Singapore	Unknown	MiSeq	4,054,129	64	154,245	63,346	38.9	3,882	65	LAIN00000000
S36	Singapore	Unknown	MiSeq	4,022,630	64	191,442	62,854	38.9	3,844	67	LAIO00000000
S46	Singapore	Unknown	MiSeq	4,043,025	70	133,482	57,758	38.9	3,875	63	LAIZ00000000

Chapter 5. Discussion

5.1 Outline of findings

The detection of species specific intrinsic oxacillinases genes of *Acinetobacter* spp. was addressed in Chapter 2 [102]. Here, a total of six intrinsic *bla*$_{OXAs}$ of non-*baumannii Acinetobacter* species were determined by the multiplex PCR. Further, the *bla*$_{OXA}$ genes and its IS element associated with OXA of *Acinetobacter* non-*baumannii* were characterised. This chapter also included a case of unique *A. pittii* isolated from Australia containing multiple carbapenemases and two other beta-lactamases [103].

The central focus of this thesis is the molecular epidemiology and genome of *A. baumannii* as described in Chapter 3 and 4. Three hundred *Acinetobacter* spp. isolates were included in the molecular epidemiology. Twenty-four *Acinetobacter* spp. isolates were genome sequenced and analysed in these two chapters.

A major finding of this thesis is that OXA-23 carbapenemase and the complete set of biofilm-producing locus or operon are the two significant contributing factors to the dominance of *A. baumannii* IC2 in a university hospital in Bangkok, Thailand (Chapter 3). The comparison of the genome data of *A. baumannii* and non-*baumannii* shows significantly higher number of antimicrobial resistance genes present amongst carbapenem-resistant isolates in comparison to the carbapenem-susceptible *Acinetobacter* spp. The detailed analysis of the resistance islands and pathogenicity islands shows that the cluster of antimicrobial resistance genes are generally concentrated in IC2 (Chapter 4). Detailed findings for each chapter of this thesis are described in the following three main sub-headings.

5.1.1 Detection of species specific oxacillinases genes within *Acinetobacter* and characterisation of pathogenic non-*baumannii Acinetobacter* spp.

To identify species specific intrinsic oxacillinases genes in *Acinetobacter* species, a multiplex PCR was developed. The multiplex PCR is able to aid in differentiation of six *Acinetobacter* species including *A. lwoffii*/ *A. schindleri* (*bla*$_{OXA\text{-}134\text{-}like}$), *A. johnsonii* (*bla*$_{OXA\text{-}211\text{-}like}$), *A. calcoaceticus* (*bla*$_{OXA\text{-}213\text{-}like}$), *A. haemolyticus* (*bla*$_{OXA\text{-}214\text{-}like}$) and *A. bereziniae* (*bla*$_{OXA\text{-}228\text{-}like}$). A total of 30 novel oxacillinases are described here. The majority of these novel *bla*$_{OXA}$ variants belonged to the *bla*$_{OXA}$ from *A. calcoaceticus*, i.e. *bla*$_{OXA\text{-}213\text{-}like}$. A novel finding was the presence of an unusual insertion

element, IS*Aba11* upstream to *bla*$_{\text{OXA-214}}$ [102]. Regardless of the presence of IS*Aba11*, the isolates remained susceptible to carbapenem.

The study of oxacillinase genes amongst isolates collected worldwide has contributed to the understanding of the acquired-types of *bla*$_{\text{OXA}}$ in *Acinetobacter* spp. other than *A. baumannii*. Here, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-40-like}}$ and *bla*$_{\text{OXA-143-like}}$ were described. *A. pittii* was the predominant species (67%) harbouring *bla*$_{\text{OXAs}}$ collected from Asia, Europe, South America and North America (Appendix A2.4) [104].

A unique case of *A pittii* ST119 harbouring *bla*$_{\text{IMP-4}}$, *bla*$_{\text{OXA-421}}$, *bla*$_{\text{OXA-96}}$ in Australia is included in this Chapter 2.2 [103]. The isolate was initially identified carbapenem resistant *A. calcoaceticus* - *A. baumannii* complex. Conventional PCR identified the *bla*$_{\text{IMP-4}}$, which is extremely uncommon amongst *Acinetobacter* spp. in Australia. Detailed molecular characterisation followed by whole genome sequencing revealed other important features of this isolate, including the presence of three other β-lactamase encoding genes, *bla*$_{\text{OXA-96}}$ (*bla*$_{\text{OXA-58-like}}$), *bla*$_{\text{CARB-2}}$ and a novel *bla*$_{\text{OXA}}$, *bla*$_{\text{OXA-421}}$. This report also shows the first description of the *bla*$_{\text{OXA-96}}$ in Australia.

Overall, this chapter has multi-faceted approaches to the understanding of non- *baumannii* *Acinetobacter* that are often overlooked due to its low prevalence in comparison to the carbapenem-resistant *A. baumannii*.

5.1.2 Predominance of international clone 2 OXA-23-producing *Acinetobacter baumannii* and insights into the genome of *Acinetobacter* spp.

Chapter 3 is the tenet of this thesis containing the detailed description of *A. baumannii* IC2 from Thailand using molecular epidemiology and the overview of the genome characteristics. The predominance of *A. baumannii* IC2 is the highlight of this chapter. *A. baumannii* IC2 was found in 80% of the 300 study isolates from Thailand. In addition to this study, the molecular characterisation of *A. baumannii* from Turkey (*n*=65) is described in Appendix 3.3 of this thesis. One hundred percent of *A. baumannii* from Turkey were OXA-23-producers. Further, 97% of the study isolates from Turkey were *A. baumannii* IC2. However, due to the main focus of this manuscript is on OXA-48-producing *Klebsiella pneumoniae*, the molecular epidemiology data of *A. baumannii* was not included in this published manuscript [105]. The published article, table of *bla*$_{\text{OXA}}$ characterisation and figure of clonal analysis are presented in Appendix 2.1, 3.3 and 3.4, respectively. These two studies showed the dominance of *A. baumannii* IC2 from both institutes in Thailand and Turkey.

Resistance to broad-spectrum aminoglycoside conferred by *armA*, i.e. 16S rRNA methylase was commonly identified amongst *A. baumannii* IC2. *A. nosocomialis* (1.3%) and *A. pittii* (0.7%) were isolated in much lower prevalence. Limited diversity was observed amongst *A. baumannii* IC2. A total of 13 representative *Acinetobacter* isolates, comprised of *A. baumannii* (*n*=11), *A. nosocomialis* (*n*=1) and *A. pittii* (*n*=1) were whole genome sequenced. Our WGS data from this manuscript are publicly available through www.ncbi.nlm.nih.gov with BioProject PRJNA255268 (Chapter 4 and Table S1).

Within this study, the carbapenemase *bla*$_{\text{OXA-66}}$ was generally associated with *A. baumannii* IC2. Three isolates were detected to have interruption of the *bla*$_{\text{OXA-78}}$ by IS*Aba19*. Interestingly, these isolates were carbapenem susceptible. Other interesting features of the *A. baumannii* IC2 were the backbone of resistance AbaR4 which was integrated into a specific genomic site, *comM* (ATPase gene). AbaR4 was commonly found in IC2, which contained *uspA* (universal stress protein A), *sup* (sulphate permease), *tet(B)* (tetracycline resistance), *strB* (aminoglycoside resistance) and *strA* (aminoglycoside resistance) including Tn*2006* that carries *bla*$_{\text{OXA-23}}$ [16]. The *armA* was located in the Tn*1548* which was outside the AbaR4 and in close proximity with two macrolide resistance genes, *mphE* and *msrE*. The set of antimicrobial resistance genes, i.e. *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-66}}$, *bla*$_{\text{ADC}}$, *bla*$_{\text{TEM-1}}$, *strA, strB, aphA1, mphE, msrE, sul2* and *tetB* represented the typical characteristics of CRAB at Siriraj Hospital, Bangkok.

Another important finding of *A. baumannii* were the biofilm-related genes. All the key biofilm-related genes in *Acinetobacter* spp, i.e. *bap, csu* locus, *bfmRS* and the *pga* locus were identified in the eight ST2 strains (IC2) of the 13 isolates being genome sequenced. In contrast, the *Acinetobacter* non-IC2 generally had one or two of the biofilm-related genes absent in comparison. This shows the association between biofilm-related genes and the persistence of IC2 in the hospital environment.

5.1.3 Genomes of *Acinetobacter baumannii* and non-*baumannii Acinetobacter*.

The genome of 21 representative isolates including 17 CRAB and 4 CSAB are described in Chapter 4. This study was the first comparative whole genome sequence analysis from multiple countries from Asia. In general, whole genome sequencing and analysis of CRAB isolates shows the dominance of OXA-23-producing *A. baumannii* IC2. The *in silico* MLST showed that 13 CRAB isolates and 2 CSAB belonged to ST2. ST1 was found in one isolate from Singapore.

The antimicrobial resistance island, AbaR4-type, which was identified within *comM* (ATPase gene) contains multiple antimicrobial resistance genes. In contrast, *comM* did not contain any antimicrobial resistance gene within carbapenem-susceptible isolates from Thailand. The AbaR4 contained genes

encoding resistance to aminoglycosides, tetracyclines and sulphonamides. In isolates positive with *bla*$_{TEM-1}$, *bla*$_{TEM-1}$ was located in a Tn*1* derived transposon within the second island, AbGRI2 together with *aacC1* and *aadA1*, gentamicin and streptomycin resistance genes, respectively.

The *bla*$_{OXA-23}$, which was located in a transposon Tn*2006*, was the most common carbapenem resistance mechanism in CRAB. The composition of Tn*2006* was identical to previously described Tn*2006* comprising of *bla*OXA-23, APTase gene, DEAE helicase gene and *yeeA*, flanked by two copies of IS*Aba1* [106]. *bla*$_{IMP-4}$ was identified in one isolate from Singapore and located in an integron class 1. The composition of integron class 1 containing *bla*$_{IMP-4}$-qacG2-*aacA4-catB3* was identical to the integron class 1 in *A. pittii* ST119 as described in Chapter 2.3 [103].

The comparative genome analysis revealed variations in the loci responsible for the surface polysaccharide (K and OC loci) and regions of T6SS and *csu* operon. The unique genetic features of our IC2 Asian isolates were observed in the present study comprising (i) intrinsic β-lactamase genes; *bla*$_{OXA-66}$, and *bla*$_{ADC-30-like}$ associated with IS*Aba1* upstream, (ii) resistance island AbaR4-(I), (iii) the OC locus type OCL1 and (iv) intact TS66 and *csu* operon regions.

5.2 General discussion and conclusion

This thesis contains the first comprehensive analysis of the molecular epidemiology and genome of *A. baumannii* from Thailand. In addition, the genome analysis of *A. baumannii* from three other countries were included and compared with the genome of *A. baumannii* from Thailand. Various novel and previously described *bla*$_{OXA}$ are idetified in this thesis. Although this is a small sample size, the correlation between *bla*$_{OXA-51-like}$ variants showed a close association with the international clone designation, which fitted well with previously described studies [107, 108]. The dissemination of OXA-23-producing *A. baumannii* by clonal expansion in Siriraj Hospital is concerning. However, the dominance of OXA-23 has been also reported elsewhere, including in Australia [29]. This clonal expansion was potentially due to environmental contamination and person-to-person contact [2].

The genome analysis of isolates showed that the two evident characteristics present in *A. baumannii* IC2 are antimicrobial resistance genes and the biofilm forming locus or operon. The exception was a carbapenem resistant *A. baumannii* from Japan (J65) which possessed *bla*$_{OXA-23}$ without a complete set of biofilm forming locus or operon. This may be an early indication of OXA-23 producing IC2's inability to disseminate in Japan. It is interesting that only Tn*2006* was found in the 21 study isolates

which had been genome sequenced. Tn*2007* and Tn*2008,* which had been reported in USA and Europe, were not found amongst our study isolates [97].

The antimicrobial susceptibility of 21 isolates showed that the majority of the isolates were susceptible to ampicillin-sulbactam. Ampicillin-sulbactam susceptible isolates generally did not possess *bla*$_{\text{TEM-1}}$. However, the high MIC to sulbactam has been associated with possession of *bla*$_{\text{TEM-1}}$ [109]. Ampicillin-sulbactam MICs of two carbapenem-resistant and five carbapenem-susceptible *Acinetobacter* spp. were within the susceptible range (Appendix 3.1). Ampicillin-sulbactam susceptibility among *A. baumannii* has also been reported in other countries [110, 111]. Therefore, this antimicrobial has been recommended as a treatment option for infections by *A. baumannii* [112]. Ampicillin-sulbactam has been successful in treating patients with carbapenem susceptible *A. baumannii* infections. However, lower success rates were reported, when this antimicrobial agent was used to treat carbapenem resistant *A. baumannii* [113].

5.3 Future directions

Studies in this thesis have expanded knowledge in important aspects of carbapenem resistance and overall whole genome sequence description of *A. baumannii* and non-*baumannii*. The dissemination of carbapenemase genes coupled by 16S rRNA methylase genes conferring aminoglycoside resistance will likely escalate. Therefore, the threat posed by limited treatment to clinically important antimicrobial agents should not be underestimated. Various regimens of combination therapy have been proposed for the treatment of CRAB infections [112]. Our findings of typical clonality and antimicrobial resistance phenotypes may facilitate justification of empirical combination therapy at Siriraj hospital.

The clonal commonality of OXA-23-producing *A. baumannii* in this study is possibly a consequence of environmental contamination and person-to-person transmission. This emphasises the importance of elimination of CRAB colonisation from the patients and hospital environment in order to reduce transmission. Disinfectants and antiseptics have been widely used to control colonisation such as chlorhexidine and propenol, PVP-iodine and tricolsan [52, 53, 114]. However, the MIC of chlorhexidine in *A. baumannii* increased after the use of chlorhexidine to control colonisation in patients [115]. This suggests that apart from active surveillance for antimicrobial resistance, continuation of surveillance for chlorhexidine including other disinfectants utilised in infection prevention schemes, may require.

Extensive analysis of the transmission of *A. baumannii* in an intensive care unit using a mathematical model has previously shown that increasing the nurse-patient ratio and improvement of environmental contamination including increasing hand washing rate resulted in the decline of *A. baumannii* colonisation [116]. Additionally, antimicrobial stewardship has significantly decreased the rate of antimicrobial resistance rates in *A. baumannii* without affecting the medical quality [117]. Taken together, a possible research area that may help control the dissemination of *A. baumannii* is to establish an effective approach in "source control" in particular to eradicate colonisation of *A. baumannii* from patients and the hospital environment based on current literature and concepts elucidated from this thesis.

To complement the infection control aspect in order to eradicate IC2, the development of effective vaccines is also required. Various attempts have been made to develop vaccines for *A. baumannii* over the past few years such as using K1 capsular polysaccharide and other novel vaccine candidates [118-120]. However, based on the recent studies to understand the surface of polysaccharides of *A. baumannii*, *A. baumannii* possessed lipooligosaccharide (LOS) and not lipopolysaccharide (LPS) [64]. Polysaccharide is highly immunogenic, in contrast to lipooligosaccharide that is not. This will be challenging in the development of potential targets of vaccines against *A. baumannii*. The genome data of this thesis, which is available publicly, may be used in finding potential vaccine candidates. Lastly, further understanding the *A. baumannii* at the proteomic level may also help in the vaccine development.

The epidemiology and molecular epidemiology data of *A. baumannii* from countries in South East Asia is currently limited to Thailand, Singapore, Malaysia, Indonesia and Vietnam [81, 121, 122]. In fact, countries in this region and the neighbouring countries, such as Myanmar, the Philippines and Papua New Guinea, which have no epidemiological data of *A. baumannii*, may have similar rates of CRAB to Thailand. In some countries, the genus and species identification of *A. baumannii* is still problematic and not implemented yet in clinical diagnostics. Therefore, an extensive surveillance and screening using molecular methods in wider regions of South East Asia should be proposed, utilising methods such as Multiplex PCR developed in this thesis which can rapidly detect and identify *Acinetobacter* spp. in this region. Overall, there are multiple aspects of *A. baumannii* the warrant further investigation to overcome the problems by *A. baumannii* in greater details.

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APPENDICES

Table A1. Molecular and characterisation of Acinetobacter study isolates from Thailand

isolate	AM	ATM	CIP	FEP	IPM	MEM	Remark	Date	Source	Ward	OXA-51	OXA-23	OXA-40	OXA-58	SAba1/OXA-5	SG	Species	ArmA
T1	R	9	R	R	R	R		Jan-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T2	R	12	R	R	R	R		Jan-10	Pleural flu	ICU	1	1	0	0	0	1	A. baumannii	1
T3	R	12	R	R	R	R		Jan-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T4	R	10	R	R	R	R		Jan-10	Sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T5	R	11	R	R	R	R		Jan-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T6	R	18	R	I	R	R		Jan-10	Sputum	ICU	1	1	0	0	0	4	A. baumannii	1
T7	R	11	R	R	R	R		Jan-10	CSF	ICU	1	1	0	0	0	1	A. baumannii	1
T8	R	9	R	R	R	R		Jan-10	Hemoculture	OPD	1	1	0	0	0	1	A. baumannii	1
T9	R	11	R	R	R	R		Jan-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T10	R	7	R	R	R	R		Jan-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T11	R	12	R	R	R	R		Feb-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T12	R	10	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T13	R	18	R	I	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	4	A. baumannii	1
T14	R	16	R	I	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	4	A. baumannii	1
T15	R	10	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T16	R	11	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T17	R	10	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T18	R	13	R	R	R	R		Feb-10	Peritoneal	ICU	1	1	0	0	0	1	A. baumannii	1
T19	R	7	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T20	R	13	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T21	R	7	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T22	R	11	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T23	R	13	R	R	R	R		Feb-10	Pus	OPD	1	1	0	0	0	1	A. baumannii	1
T24	R	7	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T25	R	14	R	R	R	R		Feb-10	Swab	Surgery	1	1	0	0	0	1	A. baumannii	1
T26	R	7	R	R	R	R		Feb-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T27	R	14	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T28	R	13	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T29	R	16	R	R	R	R		Feb-10	Urine	Medicine	1	1	0	0	0	4	A. baumannii	1
T30	R	13	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T31	R	12	R	R	R	R		Feb-10	Urine	Orthopedic	1	1	0	0	0	1	A. baumannii	1
T32	R	11	R	R	R	R		Feb-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T33	R	10	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T34	R	8	R	R	R	R		Feb-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T35	R	7	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T36	R	8	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T37	R	7	R	R	R	R		Feb-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T38	R	15	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T39	R	12	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T40	R	11	R	R	R	R		Feb-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T41	R	16	R	I	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	0
T42	R	11	R	R	R	R		Feb-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T43	R	12	R	R	R	R		Feb-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T44	R	7	R	R	I	R		Feb-10	Urine	Surgery	1	1	0	0	0	5	A. baumannii	1
T45	R	8	R	R	R	R		Feb-10	Lavage	ER	1	1	0	0	0	1	A. baumannii	1
T46	R	13	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T47	R	11	R	R	R	R		Feb-10	Tissue biop	84/3	1	1	0	0	0	1	A. baumannii	1
T48	R	7	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T49	R	10	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T50	R	11	R	R	R	R		Feb-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T51	R	9	R	R	R	R		Feb-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T52	R	10	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T53	R	13	R	R	R	R		Feb-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T54	R	10	R	R	R	R		Mar-10	Urine	Surgery	1	1	0	0	0	1	A. baumannii	1
T55	R	7	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	5	A. baumannii	1
T56	R	12	R	R	R	R		Mar-10	Sputum	MV 2]	1	1	0	0	0	1	A. baumannii	1
T57	R	9	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T58	R	9	R	R	R	R		Mar-10	Sputum	CCU	1	1	0	0	0	1	A. baumannii	1
T59	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T60	R	7	R	R	R	R		Mar-10	Hemoculture	Medicine	1	1	0	0	0	1	A. baumannii	1
T61	R	13	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T62	R	12	R	R	R	R		Mar-10	Pus	Surgery	1	1	0	0	0	1	A. baumannii	1
T63	S		R	I	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	4	A. baumannii	1
T64	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T65	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T66	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T67	R	15	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T68	R	10	R	R	R	R		Mar-10	Hemoculture	Bone Marro	1	1	0	0	0	1	A. baumannii	1
T69	R	9	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T70	R	7	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T71	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T72	S	20	R	S	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	4	A. baumannii	0
T73	R	15	R	R	R	R		Mar-10	Others	Medicine	1	1	0	0	0	1	A. baumannii	0
T74	R	7	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T75	R	12	R	I	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T76	R	8	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T77	R	15	R	R	R	R		Mar-10	Swab	Medicine	1	1	0	0	0	1	A. baumannii	1
T78	R	10	R	R	R	R		Mar-10	Hemoculture	ICU	1	1	0	0	0	1	A. baumannii	1
T79	R	9	R	R	R	R		Mar-10	Tip	ICU	1	1	0	0	0	1	A. baumannii	1
T80	R	14	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T81	R	16	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T82	R	10	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T83	R	12	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T84	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T85	R	14	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T86	R	13	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T87	R	9	R	R	R	R		Mar-10	Hemoculture	ICU	1	1	0	0	0	1	A. baumannii	1
T88	R	16	R	I	R	R		Mar-10	Hemoculture	Medicine	1	1	0	0	0	1	A. baumannii	1
T89	R	10	R	R	R	R		Mar-10	Hemoculture	ICU	1	1	0	0	0	1	A. baumannii	1
T90	R	8	R	R	R	R		Mar-10	Others	ICU	1	1	0	0	0	1	A. baumannii	1
T91	R	12	R	R	R	R		Mar-10	Sputum	CCU	1	1	0	0	0	1	A. baumannii	1
T92	R	7	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	5	A. baumannii	0
T93	R	10	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T94	R	9	R	R	R	R		Mar-10	Others	ICU	1	1	0	0	0	1	A. baumannii	1
T95	R	7	R	R	R	R		Mar-10	Sputum	Other	1	1	0	0	0	5	A. baumannii	0
T96	R	11	R	R	R	R		Mar-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T97	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T98	R	10	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T99	R	7	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	5	A. baumannii	0
T100	R	12	R	R	R	R		Mar-10	Hemoculture	Medicine	1	1	0	0	0	1	A. baumannii	1
T101	R	10	R	R	R	R		Mar-10	Sputum	Orthopedic	1	1	0	0	0	1	A. baumannii	1
T102	R	9	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T103	R	16	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T104	R	8	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T105	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T106	R	7	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T107	R	7	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T108	R	16	R	R	R	R		Mar-10	Swab	Medicine	1	1	0	0	0	1	A. baumannii	1
T109	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T110	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T111	R	12	R	R	R	R		Mar-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T112	R	11	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T113	R	7	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T114	R	8	R	R	R	R		Mar-10	Swab	ICU	1	1	0	0	0	1	A. baumannii	1
T115	R	12	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T116	R	12	R	R	R	R		Mar-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T117	R	10	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T118	R	15	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	0
T119	R	10	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T120	R	12	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T121	S	16	S	S	S	S		Mar-10	Sputum	Medicine	1	0	0	0	0	7	A. baumannii	0
T122	R	9	R	R	R	R		Mar-10	Hemoculture	Bone Marro	1	1	0	0	0	1	A. baumannii	1

T123	R	7	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T124	R	11	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T125	R	12	R	R	R	R		Mar-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T126	R	16	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T127	R	12	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T128	R	15	R	R	R	R		Mar-10	Urine	Medicine	1	1	0	0	0	4	A. baumannii	1
T129	R	12	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T130	R	9	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T131	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T132	R	9	R	R	R	R		Mar-10	Tissue biop	ICU	1	1	0	0	0	1	A. baumannii	1
T133	R	12	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T134	R	11	R	R	R	R		Mar-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T135	R	7	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T136	R	12	R	R	R	R		Mar-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T137	R	10	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T138	R	8	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T139	R	15	R	R	R	R		Mar-10	Sputum	OPD1	1	1	0	0	0	1	A. baumannii	1
T140	R	8	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T141	R	8	R	R	R	R		Mar-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T142	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T143	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T144	R	13	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T145	R	12	R	R	R	R		Apr-10	Sputum	MV 2]	1	1	0	0	0	1	A. baumannii	1
T146	R	15	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	4	A. baumannii	1
T147	R	11	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T148	R	13	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T149	S	12	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T150	R	12	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T151	R	10	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T152	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T153	R	9	R	R	R	R		Apr-10	Swab	ICU	1	1	0	0	0	1	A. baumannii	1
T154	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T155	R	7	R	R	R	R		Apr-10	Urine	Medicine	1	1	0	0	0	1	A. baumannii	1
T156	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T157	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T158	S	12	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	4	A. baumannii	0
T159	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T160	R	12	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T161	R	13	R	R	R	R		Apr-10	Sputum	MV 2]	1	1	0	0	0	1	A. baumannii	1
T162	R	16	R	I	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	4	A. baumannii	1
T163	R	13	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T164	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T165	S	11	R	R	R	R		Apr-10	Sputum	Orthopedic	1	1	0	0	0	4	A. baumannii	0
T166	R	13	R	R	R	R		Apr-10	Urine	OPD	1	1	0	0	0	1	A. baumannii	1
T167	R	7	R	R	R	R		Apr-10	Urine	84/6	0	1	0	0	0	5	A. pittii	0
T168	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T169	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T170	R	9	R	R	R	R		Apr-10	Sputum	Orthopedic	1	1	0	0	0	1	A. baumannii	1
T171	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T172	R	12	R	R	R	R		Apr-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T173	R	9	R	R	R	R		Apr-10	Aspirate	Pediatric	1	1	0	0	0	1	A. baumannii	1
T174	R	10	R	I	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T175	R	10	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T176	R	15	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T177	R	9	R	R	R	R		Apr-10	Sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T178	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T179	S	11	R	R	R	R		Apr-10	Urine	Orthopedic	1	1	0	0	0	4	A. baumannii	0
T180	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T181	R	12	R	R	R	R		Apr-10	Sputum	MV 2]	1	1	0	0	0	1	A. baumannii	1
T182	R	16	R	I	R	R		Apr-10	Sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T183	R	10	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T184	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T185	R	14	R	R	R	R		Apr-10	Hemoculture	Surgery	1	1	1	0	0	1	A. baumannii	0
T186	R	8	R	R	R	R		Apr-10	Hemoculture	Medicine	1	1	0	0	0	1	A. baumannii	1
T187	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T188	R	15	R	R	R	R		Apr-10	Sputum	OPD	1	0	1	0	0	1	A. baumannii	0
T189	R	12	R	R	R	R		Apr-10	Sputum	OPD	1	0	1	0	0	1	A. baumannii	0
T190	R	10	R	R	R	R		Apr-10	Sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T191	R	9	R	R	R	R		Apr-10	Hemoculture	OPD	1	1	0	0	0	1	A. baumannii	1
T192	R	10	R	R	R	R		Apr-10	Swab	Medicine	1	1	0	0	0	1	A. baumannii	1
T193	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T194	R	10	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T195	R	11	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T196	R	11	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T197	R	9	R	R	R	R		Apr-10	Sputum	RCU	1	1	0	0	0	1	A. baumannii	1
T198	R	7	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T199	R	7	R	R	R	R		Apr-10	Sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T200	R	8	R	R	R	R		Apr-10	Sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T201	S	16	S	S	S	S		May-10	sputum	ICU	0	0	0	0	0	all-	A. nosocomialis	0
T202	S	18	S	S	S	S		May-10	Urine	Surgery	1	0	0	0	0	12 (1kb)	A. baumannii	0
T203	S	13	S	S	S	S		May-10	sputum	Surgery	1	0	0	0	0	9	A. baumannii	0
T204	S	10	R	S	S	S		May-10	sputum	OPD	1	0	0	0	0	5	A. baumannii	0
T205	S	15	S	S	S	S		May-10	sputum	ICU	0	0	0	0	0	all-	A. nosocomialis	0
T206	S	14	S	S	S	S		May-10	sputum	ICU	1	0	0	0	0	9	A. baumannii	0
T207	S	14	S	S	S	S		Jun-10	sputum	ICU	1	0	0	1	0	9	A. baumannii	0
T208	S	16	R	S	S	S		Jun-10	sputum	Medicine	1	0	0	0	0	5	A. baumannii	0
T209	R	14	S	S	S	S	psuedo-like	Jun-10	sputum	ICU	>1kb	0	0	1	0	7	A. baumannii	0
T210	S	15	S	S	S	S		Jun-10	sputum	Medicine	0	0	0	0	0	all-	A. nosocomialis	0
T211	S	16	S	S	S	S		Jun-10	Urine	PV2	1	0	0	0	0	5	A. baumannii	0
T212	S	10	S	S	S	S		Jun-10	sputum	ICU	1	0	0	0	0	5	A. baumannii	0
T213	S	16	S	S	S	S		Jun-10	pus	N/A	1	0	0	0	0	5	A. baumannii	0
T214	S	11	R	S	S	S		Jun-10	sputum	ICU	1	0	0	0	0	5	A. baumannii	0
T215	S	22	S	S	S	S		Jun-10	sputum	Surgery	1	0	0	0	0	12 (1kb)	A. baumannii	0
T216	S	18	S	S	S	S		Jun-10	sputum	ICU	1	0	0	0	0	12 (1kb)	A. baumannii	0
T217	S	19	S	S	S	S		Jun-10	Hemoculture	Social	0	0	0	0	0	all-	A. pittii	0
T218	S	15	S	S	S	S		Jun-10	sputum	Surgery	1	0	0	0	0	10	A. baumannii	0
T219	S	18	S	S	S	S		Jun-10	sputum	Surgery	1	0	0	0	0	12	A. baumannii	0
T220	S	16	S	S	S	S		Jun-10	sputum	ICU	1	0	0	0	0	5	A. baumannii	0
T221	S	16	S	S	S	S		Jun-10	sputum	Medicine	1	0	0	0	0	12	A. baumannii	0
T222	S	15	S	S	S	S	psuedo-like	Jul-10	sputum	ICU	>1kb	0	0	0	0	7	A. baumannii	0
T223	R	10	R	R	R	R		Jul-10	sputum	N/A	1	?	0	0	0	1	A. baumannii	0
T224	S	15	S	S	S	S		Jul10	sputum	OPD	1	0	0	0	0	12	A. baumannii	0
T225	S	13	S	S	S	S		Jul-10	sputum	OPD	1	0	0	0	0	5	A. baumannii	0
T226	S	14	S	S	S	S		Jul-10	sputum	CK2	1	0	0	0	0	7	A. baumannii	0
T227	S	13	S	S	S	S	psuedo-like	Jul-10	sputum	Surgery	>1kb	0	0	0	0	7	A. baumannii	0
T228	S	13	S	S	S	S		Jul-10	sputum	ICU	0	0	0	0	0	all-	A. nosocomialis	0
T229	S	18	S	S	S	S		Jul-10	sputum	Pediatric	1	0	0	0	0	12	A. baumannii	0
T230	S	10	R	S	S	S		Jul-10	sputum	Surgery	1	0	0	0	0	5	A. baumannii	0
T231	R	8	R	S	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T232	S	11	R	R	R	R		Jun-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	0
T233	R	8	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T234	R	14	R	R	R	R		Jun-10	Urine	OPD	1	1	0	0	0	1	A. baumannii	1
T235	R	10	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T236	S	17	R	I	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	8	A. baumannii	0
T237	R	16	R	R	R	R		Jun-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T238	R	12	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T239	I	6	R	R	R	R		Jun-10	Swab	OPD	1	1	0	0	0	conclusi	A. baumannii	0
T240	S	14	I	I	I	R		Jun-10	sputum	Medicine	1	1	0	0	0	12	A. baumannii	0
T241	S	15	R	I	R	R		Jun-10	Hemoculture	ICU	1	1	0	0	0	12	A. baumannii	0

T242	R	9	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T243	R	10	R	R	R	R		Jun-10	tissue biop	ICU	1	1	0	0	0	1	A. baumannii	1
T244	R	13	R	R	R	R		Jun-10	sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T245	R	10	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T246	R	12	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T247	S	12	R	R	R	R		Jun-10	sputum	Surgery	1	1	0	0	0	12	A. baumannii	0
T248	R	12	R	R	R	R		Jun-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T249	R	17	R	R	R	R		Jun-10	Pus	OPD	1	1	0	0	0	4	A. baumannii	1
T250	R	13	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T251	R	11	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T252	R	11	R	R	R	R		Jun-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T253	R	12	R	R	R	R		Jun-10	Hemoculture	Pediatric	1	1	0	0	0	1	A. baumannii	1
T254	R	12	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T255	R	13	R	R	R	R		Jun-10	sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T256	R	13	R	R	R	R		Jun-10	sputum	CK2	1	1	0	0	0	1	A. baumannii	1
T257	R	12	R	R	R	R		Jun-10	sputum	Orthopedic	1	1	0	0	0	1	A. baumannii	1
T258	R	14	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T259	R	14	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T260	R	13	R	R	R	R		Jun-10	sputum	Pediatric	1	1	0	0	0	8	A. baumannii	1
T261	R					R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T262	R	13	R	R	R	R		Jun-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T263	R	13	R	R	R	R		Jul-10	tissue biop	OPD	1	1	0	0	0	1	A. baumannii	1
T264	R	12	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T265	R	11	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T266	R	12	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T267	S	13	R	R	R	R		Jul-10	swab	OPD	1	1	0	0	0	4	A. baumannii	0
T268	R	12	R	R	R	R		Jul-10	sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T269	R	13	R	R	R	R		Jul-10	tissue biop	Medicine	1	1	0	0	0	1	A. baumannii	1
T270	R	13	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T271	S	17	R	I	R	R	mucoid	Jul-10	urine	CK1	1	1	0	0	0	4	A. baumannii	0
T272	R	7	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T273	R	9	R	R	R	R		Jul-10	Urine	ICU	1	1	0	0	0	1	A. baumannii	1
T274	R	12	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T275	S	12	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	12	A. baumannii	0
T276	R	12	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T277	R	12	R	R	R	R		Jul-10	sputum	RCR	1	1	0	0	0	1	A. baumannii	1
T278	R	13	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T279	R	15	R	R	R	R		Jul-10	sputum	Surgery	1	1	0	0	0	4	A. baumannii	1
T280	R	9	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T281	R	13	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T282	R	6	R	R	R	R		Jul-10	sputum	OPD	1	1	0	0	0	1	A. baumannii	1
T283	S	16	R	I	I	R		Jul-10	sputum	Pediatric	1	1	0	0	0	1	A. baumannii	0
T284	R	6	R	R	R	R		Jul-10	drain	Medicine	1	1	0	0	0	5	A. baumannii	0
T285	R	7	R	R	R	R		Jul-10	sputum	Orthopedic	1	1	0	0	?	conclusi	A. baumannii	0
T286	R	14	R	R	R	R		Jul-10	sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T287	R	10	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	1
T288	R	6	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	?	conclusi	A. baumannii	1
T289	S	7	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	1	A. baumannii	0
T290	S	13	R	R	R	R		Jul-10	Body fluid	Surgery	1	1	0	0	0	1	A. baumannii	0
T291	R	13	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T292	R	20	R	S	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	4	A. baumannii	1
T293	S	14	R	R	R	R		Jul-10	Ascitic flu	ICU	1	1	0	0	0	1	A. baumannii	0
T294	S	12	R	R	R	R		Jul-10	sputum	Orthopedic	1	1	0	0	0	1	A. baumannii	0
T295	R	12	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	0
T296	S	11	R	R	R	R		Jul-10	Hemoculture	OPD	1	1	0	0	0	1	A. baumannii	1
T297	R	9	R	R	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	1
T298	R	12	R	R	R	R		Jul-10	sputum	Surgery	1	1	0	0	0	1	A. baumannii	1
T299	R	17	R	R	R	R		Jul-10	sputum	ICU	1	1	0	0	0	8	A. baumannii	1
T300	R	15	R	I	R	R		Jul-10	sputum	Medicine	1	1	0	0	0	1	A. baumannii	0

Appendix 2. Other published manuscripts during Research Higher Degree

A2.1 Evaluation of a new chromogenic medium, chromID OXA-48, for recovery of carbapenemase-producing Enterobacteriaceae from patients at a university hospital in Turkey

A2.2 Characterization of an IncN2-type *bla*$_{NDM-1}$-carrying plasmid in *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST11 and ST15 isolates in Thailand

A2.3 Predominance of VREfm ST203 subgroup in Queensland

A2.4 Worldwide dissemination of acquired carbapenem-hydrolysing class D β–lactamases in *Acinetobacter* spp. other than *Acinetobacter baumannii*

Evaluation of a new chromogenic medium, chromID OXA-48, for recovery of carbapenemase-producing Enterobacteriaceae from patients at a university hospital in Turkey

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ARTICLE

Evaluation of a new chromogenic medium, chromID OXA-48, for recovery of carbapenemase-producing Enterobacteriaceae from patients at a university hospital in Turkey

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Abstract The purpose of this study was to evaluate a new chromogenic medium, chromID OXA-48, for the isolation of carbapenemase-producing Enterobacteriaceae (CPE) directly from rectal swabs. chromID CARBA and chromID OXA-48 are two chromogenic media that have been commercialized for the isolation of CPE directly from clinical samples. Both media were evaluated alongside a broth enrichment method recommended by the CDC for isolation of CPE, with rectal swabs from 302 unique hospitalized patients at the Hacettepe University Hospital, Ankara, Turkey. A total of 33 patients (11 %) were found to be colonized with CPE using a combination of all methods, and all CPE produced OXA-48 carbapenemase. *Klebsiella pneumoniae* was by far the most dominant species of CPE and was isolated from 31 patients. Culture on chromID OXA-48 offered the highest sensitivity (75.8 %) for detection of CPE compared with the other two methods (sensitivity for both other methods was 57.6 %) and also offered the highest specificity (99.3 %). However, a combination of methods (either chromID OXA-48 plus CDC method or chromID OXA-48 plus chromID CARBA) was necessary to achieve an acceptable sensitivity (90.9 %). For isolation of CPE, in a setting where OXA-48 carbapenemase is the dominant type of carbapenemase, chromID OXA-48 is a highly useful medium but using a combination of methods is optimal for adequate detection. The combined use of two chromogenic media offered acceptable sensitivity (90.9 %) and the highest specificity (98.5 %) and also allowed for isolation of CPE within 18–20 h.

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Introduction

There is a pressing need to define robust standardized screening methods for the effective detection of carbapenemase-producing Enterobacteriaceae (CPE) in order to control their spread [1]. To address this need, the Centers for Disease Control (CDC) recommended a straightforward broth enrichment method that could be used in almost any clinical laboratory [2]. Other methods include direct culture onto chromogenic agars such as CHROMagar KPC (CHROMagar) [3], chromID CARBA (bioMérieux) [4] or *Brilliance* CRE (Oxoid) [5] or the use of direct molecular methods such as PCR [6]. Several studies have highlighted the potential difficulty in isolating Enterobacteriaceae with OXA-48-like carbapenemase as such isolates often have low carbapenem MICs and may be inhibited by some selective media that contain carbapenems [5, 7, 8]. In this study we sought to compare three methods for their ability to recover CPE from rectal swabs taken from 302 hospitalized patients attending the Hacettepe University Hospital in Ankara, Turkey. These three methods comprised: enrichment culture using 5 ml TSB plus 10-μg ertapenem as recommended by CDC [2], direct culture on an established chromogenic agar designed for detection of CPE (chromID CARBA) and finally, direct culture on a recently commercialized chromogenic agar

specifically designed for isolation of CPE that produce OXA-48 carbapenemase (chromID OXA-48).

Materials and methods

Patient samples

Rectal swabs were taken for routine screening for CPE from 302 unique patients hospitalized on eight different wards at the Hacettepe University Hospital, Ankara, Turkey between March and April 2013.

Culture of rectal swabs

chromID CARBA (reference 43861) and chromID OXA-48 agar (reference 414011) were provided by bioMérieux, La Balme-les-Grottes, France. All other materials were obtained from Oxoid, Basingstoke, UK unless stated otherwise. The material on each rectal swab was suspended in 0.5 ml of 0.85 % saline to generate a homogeneous suspension of faecal material. Aliquots of this suspension (50 μl) were used to inoculate chromID CARBA, chromID OXA-48 and 5 ml trypticase soy broth (TSB) containing a 10-μg ertapenem disc. The inoculum on the two chromogenic agars was spread to obtain isolated colonies and all media were incubated at 37 °C for 18–20 h. After incubation, the broth was mixed and a 10-μl aliquot was inoculated onto MacConkey agar, which was then incubated for 18–20 h at 37 °C. Coloured colonies on either chromogenic medium and lactose fermenting colonies on MacConkey agar were regarded as presumptive isolates of CPE in accordance with manufacturer's instructions or CDC guidelines, respectively.

Bacterial identification

To gain some insight into the selectivity of the media, all recovered isolates were identified irrespective of colony colour. Enterobacteriaceae were initially identified using API 20E (bioMérieux) and all isolates were identified by MALDI-TOF mass spectrometry (Bruker, Coventry, UK).

Phenotypic and genotypic investigation of carbapenemases

All isolates of Enterobacteriaceae recovered on any of the three media were screened for possible carbapenemase production in accordance with UK national guidelines [9] using the Rosco KPC, MBL & OXA48 confirm ID kit (Bioconnections, Knypersley, UK) in accordance with manufacturer's instructions.

All isolates showing phenotypic evidence of carbapenemase production were investigated using PCR for the five most common carbapenemase genes found in Enterobacteriaceae (i.e. those encoding for OXA-48, KPC, VIM, IMP and NDM-1) [10]. PCR amplification and sequencing of the $bla_{\text{OXA-48}}$ gene was performed on eight representative isolates of *Klebsiella pneumoniae*. The primers were forward primer (5′- GTGGCATCGATTATCGGAAT - 3′) and reverse primer (5′- CTTCTTTTGTGATGGCTTGG - 3′), which gave an amplicon of 736 bp [11]. The PCR products were sequenced by Macrogen Inc (Seoul, South Korea) using a BigDye Terminator v3.1 cycle sequencing kit with an Applied Biosystems 3730 XL sequencer. Nucleotide sequences were compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Susceptibility testing

For all isolates confirmed as CPE using PCR, susceptibility testing was performed against 18 antimicrobials by broth microdilution using commercially-prepared Sensititre trays (Trek Diagnostic Systems, East Grinstead, UK; Product: GNX2F) and results were interpreted using EUCAST breakpoints [12]. Fosfomycin susceptibility was determined by the EUCAST standardized disc diffusion method [12]. These isolates were also screened for production of extended-spectrum ß-lactamase (ESBL) and AmpC using the Rosco ESBL/AmpC Screen kit (Bioconnections, Knypersley, UK).

Clonal analysis of carbapenemase-producing *K. pneumoniae*

All isolates of carbapenemase-producing *K. pneumoniae* were analysed for their clonal relationship using semi-automated rep-PCR (Diversilab; bioMérieux, Oakleigh, Australia). DNA preparation and PCR amplification and analysis were performed as described previously [13].

Re-inoculation of CPE on to the test media

All confirmed isolates of CPE were re-inoculated (in pure culture) onto all three media (i.e. TSB plus ertapenem and both chromogenic agars). This was performed using an inoculum of approximately 100 CFU (obtained via serial dilutions in 0.85 % saline) and processed as described above. The re-inoculation of isolates was performed in duplicate on separate occasions.

Statistical analysis

Differences between the efficiencies of the two chromogenic media for isolation of carbapenemase-producing Enterobacteriaceae were compared using McNemar's test with the continuity correction applied. The dendrogram representing clonal analysis of isolates was generated using

Pearson's correlation with cut-off similarities of 95 % for isolates assigned to the same clone.

Results

Comparison of culture methods for detection of patients colonized with CPE

A total of 33 patients (11 %) were found to be colonized with CPE out of 302 distinct patients who were screened. All isolates of CPE were confirmed as harboring OXA-48 carbapenemase as confirmed by both phenotypic testing and PCR. No other carbapenemases were detected in the isolates of Enterobacteriaceae. Table 1 shows the sensitivity of each method (and combinations of the three methods) for detection of colonized patients.

Among the OXA-48 producers, *Klebsiella pneumoniae* was by far the most dominant species and was isolated from 31 patients. One patient was colonized with *Escherichia coli* only, one patient was colonized with *Enterobacter cloacae* only and one patient was colonized with both *K. pneumoniae* and *E. coli* (all with OXA-48 carbapenemase). *E. coli* produced red colonies on both chromogenic media whereas *K. pneumoniae* and *E. cloacae* formed blue colonies on chromID OXA-48 and green colonies on chromID CARBA. Culture on chromID OXA-48 was more sensitive than any other single method for isolation of CPE, although this was not statistically significant (P=0.2) and still only allowed detection of 75.8 % of colonized patients. However, using a combination of chromID OXA-48 with either of the other two methods allowed for the detection of 90.9 % of colonized patients (Table 1).

Table 2 documents the growth of other isolates that were recovered on the three media. Other than CPE, the most common other species recovered on all three media was *Acinetobacter baumannii* with 65 isolates recovered from 65 patients. All isolates of *A. baumannii* formed colorless colonies on both chromogenic agars or were non-lactose fermenting on MacConkey agar and were therefore distinct from isolates of CPE.

Susceptibility of OXA-48 producing *K. pneumoniae*

On examination of the isolates from different media, it was apparent that different colony variants of *K. pneumoniae* were present within some samples suggesting that individual patients might be colonized with more than one strain of *K. pneumoniae*. Such variation related to colony size and particularly variations in colour due to the strength of chromogenic reactions. This was supported by antimicrobial susceptibility testing, which revealed the presence of *K. pneumoniae* isolates with different susceptibility patterns within a single patient sample. In total, 49 isolates that were phenotypically distinct (on the basis of their antibiogram) were recovered from the 31 patients colonized with this species. In accordance with EUCAST criteria, all 49 isolates of *K. pneumoniae* were non-susceptible to co-trimoxazole, piperacillin-tazobactam, ticarcillin-clavulanate and ertapenem. Susceptibility to other agents was as follows: imipenem (59 %), meropenem (55 %), doripenem (47 %), cefepime (33 %), cefotaxime (16 %), aztreonam (29 %), ceftazidime (29 %), tigecycline (100 %), amikacin (98 %), gentamicin (53 %), tobramycin (8 %), colistin (47 %), levofloxacin (31 %) and ciprofloxacin (2 %). In accordance with criteria proposed by Barry et al. [14], 96 % of isolates of *K. pneumoniae* were susceptible to fosfomycin. Fifty-three percent of isolates co-produced ESBL and a further 16 % co-produced ESBL and AmpC ß-lactamase. A more detailed analysis of carbapenem susceptibility is provided in Table 3.

Re-inoculation of OXA-48 producers onto test media

All three media were re-challenged with a low inoculum (100 CFU) of all recovered isolates of CPE (n=52). These included one *E. cloacae* isolate, two *E. coli* isolates and 49 isolates of *K. pneumoniae*. All isolates of CPE grew (and produced expected coloration) on chromID OXA-48 whereas only 31 % were detected using chromID CARBA and 69 % were recovered following subculture of TSB plus ertapenem. There was a clear correlation between a low carbapenem minimum inhibitory concentration (MIC) and failure to grow on these media. For example, isolates that failed to grow in

Table 1 Total number of colonized patients detected by each method and by combinations of methods

Method	n	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Total	33				
CDC method	19	57.6	95.2	59.4	94.8
chromID OXA-48	25	75.8	99.3	92.6	97.1
chromID Carba	19	57.6	98.9	86.4	95
chromID OXA-48 plus CDC method	30	90.9	94.8	68.2	98.8
chromID OXA-48 plus chromID Carba	30	90.9	98.5	88.2	98.9
chromID Carba plus CDC method	25	75.8	94.4	62.5	96.9

PPV positive predictive value, *NPV* negative predictive value

Table 2 Non-CPE isolated from 302 non-duplicate specimens using three culture methods

Isolates	All	CDC method	chromID OXA-48	chromID Carba
Acinetobacter baumannii	65	25	47	50
Enterobacter aerogenes	1	1	0	0
Enterobacter cloacae	1	1	0	0
Escherichia coli	6	5	2	1
Klebsiella pneumoniae	11	9	1	1
Klebsiella oxytoca	3	2	1	0
Pseudomonas aeruginosa	5	2	1	3
Stenotrophomonas maltophilia	1	0	0	1
Total		45	52	56
Total false positives[a]		18	4	2

[a] In accordance with manufacturer's instructions and the CDC protocol, false positive colonies only include colored colonies on the two chromogenic media or lactose fermenters on MacConkey agar

TSB plus ertapenem typically had an ertapenem MIC of 2–4 mg/L whereas most of the isolates that were able to grow showed MICs of >4 mg/L. Similarly, growth on chromID CARBA at low inocula was only achieved by isolates with a meropenem MIC of at least 4 mg/L whereas meropenem susceptible isolates (MIC≤2 mg/L) were all inhibited when inocula of 100 CFU were re-inoculated onto chromID CARBA.

Clonal analysis of OXA-48-producing *K. pneumoniae* and sequencing of bla_{OXA-48}

All 49 isolates of *K. pneumoniae* were subjected to typing using rep-PCR and the results are shown in Fig. 1. The results indicate the presence of at least four clonal types of *K. pneumoniae* detected within patients at this hospital. The bla_{OXA-48} gene was sequenced for eight isolates, comprising two isolates from each of four distinct clusters. The gene sequences of these representative isolates showed 100 % homology to the published sequence for bla_{OXA-48} (GenBank accession number: AY236073).

Discussion

Previous studies have established that OXA-48 is the most frequently encountered carbapenemase in Enterobacteriaceae in Turkey [15]. *K. pneumoniae* is reported to be the dominant host species and outbreaks of OXA-48 producing *K. pneumoniae* have been documented [16]. There is no accepted 'gold standard' method for the detection of CPE from clinical specimens. Molecular methods are available [6] but they fail to provide any information regarding the host species or its susceptibility. Wilkinson et al. [5] examined the sensitivity and specificity of the CDC broth method with a collection of 130 CPE and 70 Enterobacteriaceae with either ESBL or AmpC ß-lactamases. They reported that ertapenem offered a superior sensitivity to meropenem as a selective agent but, even with ertapenem, 22 % of CPE were inhibited at an inoculum level of 100 CFU/ml. In this study we have shown that the CDC broth method failed to recover CPE from 42.4 % of colonized patients. Moreover the positive predictive value of the method was also limited (59.4 %) as many of the Enterobacteriaceae that were recovered using the broth method did not turn out to harbor carbapenemases (see Tables 1 and 2).

Chromogenic culture media are useful as screening tools for the isolation of antimicrobial-resistant bacteria including CPE. CHROMagar KPC (also available as pre-poured plates under the 'Colorex' brand) was the first commercially available medium designed for selective isolation of CPE. In early studies in Greece [17] and Israel [18], CHROMagar KPC was shown to have good performance when compared with MacConkey-based media supplemented with carbapenems.

Table 3 Carbapenem susceptibility data for *Klebsiella pneumoniae* isolates (n=49) with OXA-48 carbapenemase

Carbapenem	S / I / R[a]	%S	%I	%R	MIC50 (mg/L)	MIC90 (mg/L)	MIC range (mg/L)
Doripenem	≤1 / 2 / >2	47	20	33	2	>2	0.25 – >2
Ertapenem	≤0.5 / 1 / >1	0	2	98	>4	>4	1 – >4
Imipenem	≤2 / 4–8 / >8	59	12	29	2	>8	≤1 – >8
Meropenem	≤2 / 4–8 / >8	55	10	35	2	>8	≤1 – >8

[a] Susceptible (S), Intermediate (I) and Resistant (R) as defined using EUCAST criteria

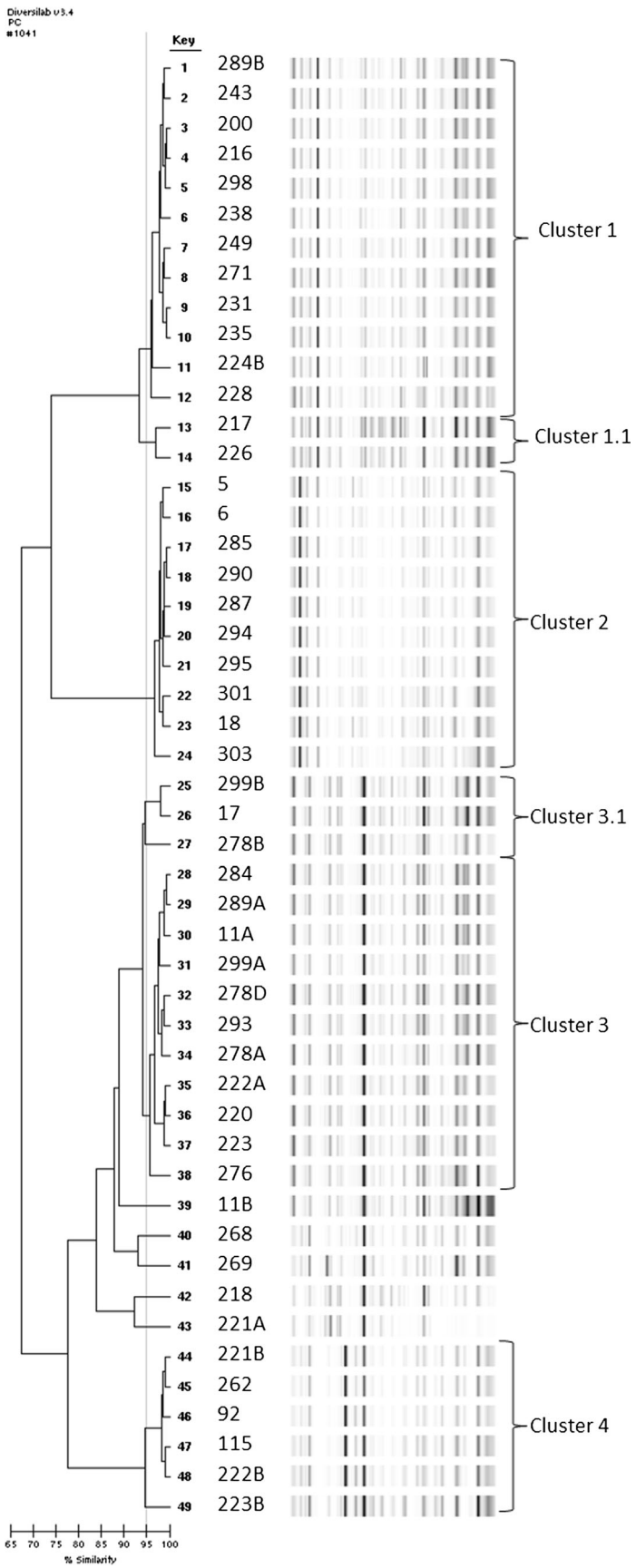

Fig. 1 Rep-PCR analysis of 49 phenotypic variants of OXA-48-producing *K. pneumoniae*

Subsequent studies showed that CPE with low carbapenem MICs (e.g. some strains with bla_{NDM-1}) may not grow on this medium, particularly at low inocula [19, 20]. chromID CARBA showed a superior performance to Colorex KPC for the isolation of CPE with NDM-1 enzyme in a study in Pakistan [20] and was superior to the CDC broth method in a report from Greece [4]. chromID CARBA was also superior to *Brilliance* CRE in two further studies in Pakistan, although the authors speculated that the selectivity of *Brilliance* CRE may have been compromised during transportation of the medium from Europe to Pakistan [21, 22]. For all of these media, a potential weakness is their limited ability to support the growth of Enterobacteriaceae with OXA-48 carbapenemase as such isolates commonly have low MICs to carbapenems [5, 7]. To address this issue, Nordmann et al. developed 'SUPERCARBA' medium, a non-chromogenic (Drigalski-based) agar medium containing a low concentration of ertapenem (0.25 mg/L), designed to accommodate the isolation of CPE with OXA-48 carbapenemase [23]. The medium has a limited shelf life of 7–10 days [7] and to our knowledge is not yet commercially available. Girlich et al. [24] evaluated SUPERCARBA for the isolation of OXA-48 producers from 77 patients hospitalized in Morocco and reported an identical sensitivity to *Brilliance* CRE but a higher specificity for SUPERCARBA (98.5 % vs. 86.6 %). We have noted only one previous report of the performance of chromID OXA-48 and the study involved inoculation of a large collection of CPE (and other bacteria) at various inocula onto chromID CARBA, SUPERCARBA and chromID OXA-48 [25]. Rectal swabs and/or stool samples (n=130) from non-colonized patients were also tested to evaluate specificity. The authors concluded that chromID OXA-48 was as sensitive for detection of OXA-48 producers as the SUPERCARBA medium, but with a higher specificity [25].

To our knowledge this is the first report that has evaluated chromID OXA-48 with a patient population that has colonization with CPE. Although chromID CARBA has proven to be highly effective in other studies, we have demonstrated its limited efficacy in a setting where OXA-48 is the dominant carbapenemase type. These findings are not unexpected and this limitation is stated in the manufacturer's product information. As noted by Girlich et al., chromID CARBA shows a weak sensitivity for detection of OXA-48 producers, but is "a powerful tool for detection of all other classes of CPE" [25]. Our study provides evidence that chromID OXA-48 is a highly useful medium for detection of OXA-48 producing Enterobacteriaceae from colonized patients and is superior to the CDC broth-based method. Twenty-five out of 33 patients (75.8 %) were detected using this medium alone and it was subsequently demonstrated that all CPE (recovered by any of the three methods) were able to grow on this medium using a low inoculum (100 CFU). This suggests that failure to isolate CPE on chromID OXA-48 was probably attributable to either a very low amount of CPE in the sample or overgrowth by other bacteria. When used with chromID CARBA, this combination of media potentially offers a highly effective solution for detection of Enterobacteriaceae with any commonly encountered carbapenemase.

One potential disadvantage of the use of chromogenic media is the increased cost to the laboratory, as they are invariably more expensive than conventional media. In May 2014, the UK list price (without tax) for materials only for the CDC method was £1.33 (1.7 EUR / 2.23 USD) whereas the combined cost for purchase of both chromID CARBA and chromID OXA-48 was £3.16 (3.9 EUR / 5.3 USD). Alternatively, the manufacturer has recently commercialized chromID CARBA SMART, which includes both media in a single Petri dish in the form of a bi-plate (£2.44 / 3.0 EUR / 4.1 USD). The increased cost will need to be weighed against the increased labour time for subculture of enrichment broths in the CDC method (and the subsequent delay in isolating CPE) and, most importantly, the relative overall effectiveness of the methods.

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Characterization of an IncN2-type *bla*$_{NDM-1}$-carrying plasmid in *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST11 and ST15 isolates in Thailand

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Sir,

The prevalence of New Delhi metallo-β-lactamase-1 (NDM-1), encoded by the *bla*$_{NDM-1}$ gene, has been increasing among various Gram-negative bacteria.[1] *bla*$_{NDM-1}$ has been shown to reside in various plasmid incompatibility (Inc) types.[1] Recently, a new Inc type, IncN2, has been identified in *Escherichia coli* and *Klebsiella pneumoniae* and proposed to acquire the *bla*$_{NDM-1}$-carrying region by transposition.[2,3] In Thailand, NDM-1 producers have become an emerging issue,[4] and yet the characteristics of the *bla*$_{NDM-1}$-carrying plasmid have not been elucidated. Here we report the genetic study of plasmids harbouring *bla*$_{NDM-1}$ from domestic isolates.

We examined three clinical isolates, one *E. coli* (ECS01) and two *K. pneumoniae* (KPS01 and KPS03), which were obtained from urine samples of independent Thai patients at two unrelated hospitals during June to August 2012 (IRB approval no. Si 454/2009). No previous hospitalization, antimicrobial exposure or healthcare-related history was documented for any patient. MIC values were determined using Etest (bioMérieux, France). According to the CLSI guideline,[5] all isolates were resistant to all tested β-lactam agents, including cefoxitin, ceftazidime, imipenem, meropenem, doripenem and piperacillin/tazobactam, as well as ciprofloxacin with high MIC values (Table 1). Both *K. pneumoniae* isolates were resistant to gentamicin, but only KPS03 was resistant to amikacin. *E. coli* ECS01 remained susceptible to both gentamicin and amikacin. Multilocus sequence typing was performed to assign sequence type (ST) using primers and amplification conditions as recommended for *E. coli* (http://mlst.ucc.ie) and *K. pneumoniae* (http://www.pasteur.fr/mlst). *E. coli* ECS01 belonged to ST131 and *K. pneumoniae* KPS01 and KPS03 were ST11 and ST15, respectively.

bla$_{NDM-1}$ was identified on a plasmid, extracted by alkaline lysis from these isolates by PCR sequencing according to the protocol reported previously.[5] The Inc type of *bla*$_{NDM-1}$-carrying plasmids was determined by multiplex PCR-based replicon typing using primers and conditions as previously published.[6] Forward and reverse primers to target the IncN2 variants were designed as N2-F: 5′-TAGCCTTCGGACAGGGTGAG-3′ and N2-R: 5′-ACGTTCGCCTGGATTTCATC-3′, respectively. All *bla*$_{NDM-1}$-carrying plasmids were matched with the IncN2-type plasmid. The *bla*$_{NDM-1}$-carrying plasmids were determined for their transferability by electroporation using *E. coli* TOP10 (Invitrogen, USA) as described elsewhere. The NDM transformants generated from ECS01, KPS01 and KPS03 parents were designated as ECS01-NDM, KPS01-NDM and KPS03-NDM, respectively. The *bla*$_{NDM-1}$-carrying plasmids from each strain were designated as pNDM-ECS01, pNDM-KPS01 and pNDM-KPS03, respectively. Southern blotting hybridization using a specific *bla*$_{NDM-1}$ probe illustrated that a *bla*$_{NDM-1}$-carrying plasmid, approximately 40 kb in size, was presented in both parents and transformants (data not shown). Transformants showed decreased susceptibility to all tested β-lactam agents, including carbapenems, but remained susceptible to gentamicin and amikacin (Table 1).

To further characterize the genetic structure of the *bla*$_{NDM-1}$-carrying plasmid, pNDM-ECS01 was selected for whole DNA sequencing using the Nextera DNA library kit (Illumina, USA) according to the manufacturer's directions, and data were generated on MiSeq (Illumina). Annotation and sequence analysis were performed using CLC Genomics Workbench (version 6.5.1). The plasmid was 41 190 bp and consisted of 50.8% GC content. Fifty-six open reading frames were predicted encoding 40 coding sequences for known proteins, 3 truncated proteins and 13 hypothetical proteins (see Table S1, available as Supplementary data at *JAC* Online). Our plasmid was most closely related to the 41 187 bp identical plasmids pTR3 and pTR4 (GenBank numbers JQ349086 and JQ349085, respectively), which were recently reported as IncN2-type *bla*$_{NDM-1}$-carrying plasmids in *K. pneumoniae* ST1 and ST273 from two patients in Singapore,[3] except for three nucleotide insertions, C, A and T, at positions 1152, 9230 and 41119, respectively, relative to the start of *repA* gene. In addition, pNDM-ECS01 was also closely related to the 35 947 bp p271A, another IncN2-type *bla*$_{NDM-1}$-carrying plasmid identified in an *E. coli* ST101 isolate from Australia.[7] A 5243 bp region at the position 3492–8734 on pNDM-ECS01 was absent in p271A. This region contained the conserved upstream repeat-controlled

Table 1. MICs of various antimicrobial agents for ECS01, KPS01 and KPS03 and their corresponding bla_{NDM-1} transformants

Antimicrobial agent	MIC (mg/L)						
	ECS01 (parent)	ECS01-NDM-1 (transformant)	KPS01 (parent)	KPS01-NDM-1 (transformant)	KPS03 (parent)	KPS03-NDM-1 (transformant)	TOP10 (host)
Cefoxitin	≥256	≥256	≥256	≥256	≥256	≥256	2
Ceftazidime	≥256	8	≥256	6	≥256	≥32	0.064
Piperacillin/tazobactam	≥256	≥256	≥256	≥256	≥256	≥256	1.5
Imipenem	≥32	24	≥32	16	≥32	≥32	0.023
Meropenem	≥32	16	≥32	≥32	≥32	≥32	0.023
Doripenem	≥32	8	≥32	6	≥32	≥32	0.032
Ciprofloxacin	≥32	0.004	≥32	0.004	≥32	0.003	0.064
Gentamicin	1	0.25	64	0.25	≥256	0.25	0.125
Amikacin	2	1.5	4	1.5	≥256	1.5	1.5

regulon normally found in IncN plasmid and was related to bacterial conjugation efficiency. The remaining pNDM-ECS01 shared 99.98% homology to p271A. The complete DNA sequence of pNDM-ECS01 was deposited to GenBank database (KJ413946).

NDM-1 producers have gained serious attention due to their high-level resistance to carbapenems. Genetic studies have shown that bla_{NDM-1} is commonly located on a plasmid that could be easily disseminated via horizontal transfer. We report here the characterization of IncN2-type bla_{NDM-1}-carrying plasmids from clinical isolates in Thailand. Unlike the previous reports, this study first demonstrated the acquisition of IncN2-type plasmid-containing bla_{NDM-1} in highly virulent *E. coli* ST131 and outbreak-related drug-resistant *K. pneumoniae* ST11 and ST15 clones.[8–10] The first IncN2-type bla_{NDM-1}-carrying plasmid from a patient in Australia had a link to the Indian subcontinent since he was transferred from a hospital in Bangladesh.[7] Our cases and cases in Singapore had no link to the Indian subcontinent and indeed had no history of traveling abroad. This suggests the potential for international multiclone spread of bla_{NDM-1} gene in this plasmid backbone. This should be an alert for continuous multinational surveillance of bla_{NDM-1}-carrying isolates to appropriately control these highly resistant bacteria.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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High tobramycin serum concentrations after tobramycin inhalation in a child with renal failure

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Sir,

Inhaled tobramycin is used to treat chronic lung infections caused by *Pseudomonas aeruginosa* in cystic fibrosis. The advantages of inhalation over parenteral administration are direct endobronchial delivery and minimal systemic toxicity. Tobramycin inhalation is considered safe and effective.[1] Serum levels in cystic fibrosis patients after inhalation are generally low (<1 mg/L),[2,3] without evidence for drug accumulation in serum.[2] Therefore, routine monitoring of systemic tobramycin levels after inhalation is not indicated.

We report elevated serum tobramycin concentrations in a child with renal failure receiving inhaled tobramycin.

An 11-year-old child was admitted to a paediatric intensive care unit for an out-of-hospital cardiac arrest. Resuscitation was complicated by bilateral pneumothorax. Extracorporeal membrane oxygenation was started on admission and continued for 4 days. Rhabdomyolysis on day 2 caused acute renal failure with oliguria. Continuous veno-venous haemofiltration (CVVH) was started on day 6. On day 8, atelectasis of the right superior lobe was detected. The patient received antimicrobials to successfully treat *Staphylococcus aureus* ventilator-associated pneumonia and systemic *Candida albicans* infection. In the fourth week intravenous ciprofloxacin was commenced to treat *P. aeruginosa* pneumonia. Because of increased diuresis and severe clotting of the line, CVVH was discontinued in the fifth week. The subsequent rise in creatinine decreased spontaneously after 5 days. One day after CVVH discontinuation, tobramycin for inhalation (TOBI®, Novartis Pharma, Basel, Switzerland) at 300 mg twice daily was started, delivered by a vibrating mesh nebulizer (Aeroneb® Pro, Aerogen, Galway, Ireland), because of increased respiratory distress with oxygen requirement, fever and elevated C-reactive protein levels. Inhalation was preferred to intravenous infusion because of renal impairment. Ciprofloxacin was switched to meropenem because of resistance of *P. aeruginosa*. In the sixth week of admission a CT scan showed empyema, for which thoracoscopic surgery and drainage was performed.

Seven days after starting tobramycin for inhalation the tobramycin serum level (13.8 mg/L 6 h after administration) was interpreted as a sampling error. The test was repeated after the weekend on day 10. The concentrations were 17.9 mg/L (trough) and 17.1 mg/L (1 h after administration). Tobramycin was discontinued on the 11th day. Concentrations after the last dose were 14.1 mg/L (after 1 h) and 13.4 mg/L (after 6 h). Five days after discontinuation the levels were <0.5 mg/L and renal function improved accordingly (Figure 1). The patient's respiratory condition gradually improved and subsequent cultures were negative. The patient recovered and was discharged to a rehabilitation centre 3 months after hospital admission. We obtained informed consent from both patient and parents to publish this report.

Several possible causes for the high tobramycin levels were considered. Erroneous substitution of inhalation for intravenous therapy and medication with probable cross-reactivity of the tobramycin immunoassay were excluded. Skin contamination of fingerprick blood following nebulization of tobramycin[4] was excluded because only arterial blood was drawn.

A few case reports have described patients with renal dysfunction receiving tobramycin inhalation with high trough levels of 10.6,[5] 8.8,[6] 19.5,[7] 2.5[8] and 13.4 mg/L[9] and a peak level of 2.1 mg/L.[10] They received 80–300 mg twice daily,[5] 300 mg twice daily[6–8,10] or 600 mg twice daily[9] for 5–25 days. Four patients were ventilated,[5,8–10] one had bronchopulmonary dysplasia,[5] one was a lung transplant recipient[6] and one had bronchiectasis.[7] Two patients developed vestibular injury[6,7] and one got hearing difficulties.[9] We

Predominance of VREfm ST203 subgroup in Queensland

Sir,

The molecular epidemiology of *Enterococcus faecium* in an Australian setting has recently been described for the first time.[1] Johnson *et al.* described the epidemiology of 85 *E. faecium* isolates in blood culture over a 12 year period at a single institution in Victoria, Australia (Austin Health, Melbourne).[1] This comprised 34 vancomycin resistant *E. faecium* (VREfm) and 51 vancomycin susceptible *E. faecium* (VSEfm) isolates. They defined 17 different sequence types (STs) amongst 85 *E. faecium* isolates using multilocus sequence typing (MLST) and found three dominant STs (ST17, ST252 and ST203). Amongst the VREfm isolates, all but one carried the resistance gene, *vanB*.[1] ST17, the putative founder of clonal complex 17 (CC17), was stable and predominated in VREfm and VSEfm for the first 10 years of the 12 year study period. From 2007 to 2009, a significant increase in VREfm bacteraemia was identified.[1] The predominant ST was ST203, accounting for 76% and 81.8% of VREfm bacteraemia isolates in 2007 and 2009, respectively. ST203 is a double-locus variant of ST17 and both STs belong to CC17.

We have investigated 765 VREfm isolates (39 clinical and 726 screening isolates) from hospitalised patients across 26 hospitals in Queensland, collected from January to November 2010. *Van* genotyping was performed on all isolates. A total of 758 isolates (99.1%) had a *vanB* genotype while seven isolates were positive for *vanA*. Ninety-one *vanB* VREfm were selected to study molecular epidemiology using repetitive polymerase chain reaction (PCR) (DiversiLab; bioMérieux, France), comprising both clinical ($n = 39$) and screening ($n = 62$) specimens. Results revealed that the majority of isolates from Queensland ($n = 88$) were very closely related (>92% similarity).

Fourteen isolates from this major group were further analysed by high resolution melting (HRM) genotyping as described.[2] Four melting types (MelTs) were identified, MelT11 ($n = 1$), MelT34 ($n = 1$), MelT121 ($n = 11$) and one novel MelT. MelT11, MelT34 and MelT121 represent STs which are clustered in CC17. MelT11 and MelT121 include various STs from each subgroup founded by ST17 and ST203, respectively. MelT34 incorporated multiple subgroups of CC17 as well as the singleton ST51 (Table 1). MLST was performed in all 14 isolates as previously described;[3] of these, 12 isolates were ST203. The results from MLST were correlated with HRM genotyping except the novel MelT that was identified as ST203 (Table 1).

In conclusion, our results indicate that 88 of 91 isolates (97%) were closely related. HRM genotyping and MLST of representative isolates from this cluster revealed that ST203 was predominant. This suggests that ST203 may be responsible for VREfm in Queensland. These findings correspond with what was found by Johnson *et al.* The same ST causing outbreaks in two geographically non-contiguous states suggests that other regions in Australia may be similarly affected. ST203 has also been reported in Korea, Japan China, Germany, Denmark, The Netherlands and Serbia (http://efaecium.mlst.net/). Interestingly, ST203 isolates reported from these countries almost entirely possessed the *vanA* gene. On the contrary, the majority of isolates from Victoria and Queensland possessed *vanB*. This highlights that CC17, especially ST203, has a great ability for hospital adaptation. CC17 has caused outbreaks in multiple continents including Australia.[4] Further study and surveillance of this subgroup is necessary to understand its persistence in the hospital environment.

Table 1 Correlation of MelT with ST

MelT	Possible ST*	CC
11	**17**, 63, 103, 180, 187, 234, 267, 295, 307, 308, 357, 460, 475, 480, 538, 543, 554, 578, 584	17
34	49, 51, 177, 232, **341**, 547, 548, 556	17
121	**203**, 365, 412, 478, 483, 577	17

* Generated by *Enterococcus faecium* MLST and MelT key as described by Tong *et al.*[2] and found at http://menzies.edu.au/node/43174.
Bold type indicates ST identified by MLST.
CC, clonal complex; MelT, melting type; MLST, multilocus sequence typing; ST, sequence type.

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Short communication

Worldwide dissemination of acquired carbapenem-hydrolysing class D β-lactamases in *Acinetobacter* spp. other than *Acinetobacter baumannii*

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ABSTRACT

The aim of this study was to identify acquired OXA-type carbapenemases in *Acinetobacter* spp. other than *Acinetobacter baumannii*. From a total of 453 carbapenem-susceptible and -resistant *Acinetobacter* isolates collected worldwide, 23 were positive for bla_{OXA} genes by multiplex PCR. These isolates were identified as *Acinetobacter pittii* ($n = 18$), *Acinetobacter nosocomialis* ($n = 2$), *Acinetobacter junii* ($n = 1$) and *Acinetobacter* genomic species 14TU/13BJ ($n = 2$). The bla_{OXA} genes and associated insertion sequence (IS) elements were sequenced by primer walking. In 11 of these isolates, sequencing of the PCR products revealed that they were false-positive for bla_{OXA}. The remaining 12 isolates, originating from Europe, Asia, South America, North America and South Africa, harboured OXA-23 ($n = 4$), OXA-58 ($n = 5$), OXA-40-like ($n = 1$) and OXA-143-like ($n = 1$); one *A. pittii* isolate harboured both OXA-23 and OXA-58. IS elements were associated with bla_{OXA} in 10 isolates. OXA multiplex PCR showed a high degree of false-positive results (47.8%), indicating that detection of bla_{OXA} in non-*baumannii Acinetobacter* spp. should be confirmed using additional methods.

1. Introduction

Carbapenem-hydrolysing class D β-lactamases, also known as oxacillinases (OXA), are the most commonly reported carbapenem resistance determinants in *Acinetobacter* spp., particularly in *Acinetobacter baumannii*. In the genus *Acinetobacter*, various intrinsic OXA have already been identified, including OXA-23-like in *Acinetobacter radioresistens*, OXA-51-like in *A. baumannii*, OXA-134-like in *Acinetobacter lwoffii*, OXA-211-like in *Acinetobacter johnsonii*, OXA-213-like in *Acinetobacter calcoaceticus*, OXA-214-like in *Acinetobacter haemolyticus* and OXA-228-like in *Acinetobacter bereziniae* [1]. The commonest acquired subclass in *A. baumannii* is OXA-23-like, which is the intrinsic OXA of *A. radioresistens*, and can be encoded on plasmids or on the chromosome. Other acquired OXA subclasses, mainly encoded on plasmids in *A. baumannii*, include OXA-40-like, OXA-58-like, OXA-143-like and OXA-235-like, but their natural hosts are currently unknown [2]. Although OXA are weak carbapenem hydrolysers, they can confer resistance in the presence of additional resistance mechanisms (e.g. altered permeability) and when overexpressed, typically mediated through promoters provided by insertion sequence (IS) elements (OXA-40 and OXA-143 appear to be exceptions to this) [3]. The association with IS elements and frequent plasmid location highlight the potential of bla_{OXA} genes to spread within the genus *Acinetobacter* via transposition events and horizontal gene transfer. Several acquired OXA subclasses have already been detected in various *Acinetobacter* spp. mainly originating from European and Asian countries [4,5].

The aim of this study was to characterise the acquired bla_{OXA} genes detected by multiplex PCR in *Acinetobacter* spp.

2. Materials and methods

2.1. Bacterial isolates, bla_{OXA} screening, species identification and carbapenem susceptibility

A total of 453 clinical *Acinetobacter* isolates other than *A. baumannii* collected from 141 centres in Europe, the Americas, Southeast Asia and South Africa as part of the Tigecycline Evaluation and Surveillance Trial (TEST) and the German Tigecycline Evaluation and Surveillance Trial (G-TEST) studies were screened for the presence of bla_{OXA} genes [6,7]. Most of the isolates were members of

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the *A. baumannii* group, i.e. they were *Acinetobacter nosocomialis* or *Acinetobacter pittii*. A subset of carbapenem-resistant isolates were further tested for the presence of OXA-235 [2]. Species identification was performed using *gyrB* PCR and *rpoB* sequencing as previously described [8,9]. Clonal relatedness was investigated by repetitive sequence-based PCR (rep-PCR) (DiversiLab; bioMérieux, Nürtingen, Germany) [6]. Imipenem and meropenem susceptibility were determined by Etest (bioMérieux).

2.2. Sequencing of bla_{OXA} and associated insertion sequence elements

The bla_{OXA} genes and surrounding areas were amplified and sequenced by primer walking. $bla_{OXA\text{-}143\text{-}like}$ was amplified using primers 5′-CATCTCGGTAAACAGTCGAT-3′ and 5′-TTAATCCCCCT-CATTGAACT-3′. As flanking primers for $bla_{OXA\text{-}40\text{-}like}$ failed to amplify the gene, OXA-40-like specific primers (5′-ATGAAAAAATT-TATACTTCC-3′ and 5′-TTAAATGATTCCAAGATTTTC-3′) were used for partial sequencing. To investigate the association of $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ with IS*Aba1* or IS*Aba3*, respectively, PCR sequencing was performed using previously published primers [10,11]. In addition, inverse PCR was performed to sequence downstream of $bla_{OXA\text{-}23}$. Briefly, plasmids were extracted using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and were digested with *Bam*HI, *Eco*RI and *Eco*RV (New England BioLabs, Frankfurt am Main, Germany), respectively. Then, 5 μL of the heat-inactivated restriction reaction was self-ligated using Quick Ligase (New England BioLabs), amplified by PCR and sequenced using inverse primers OXA-23inv_F (5′-TTGGGCAATGGATATAAAAC-3′) and OXA-23inv_R (5′-TAGAGGCTGGCACATATTCT-3′).

2.3. Characterisation of false-positive bla_{OXA} PCR products

A subset of isolates amplified a PCR product for OXA-23-like and OXA-58-like in the multiplex format but failed to amplify a product using flanking primers (see Section 2.2) and bla_{OXA}-specific primers (OXA-23-like, 5′-ATGAATAAATATTTTACTTGCTATG-3′ and 5′-TTAAATAATATTCAGCTGTT-3′; and OXA-58-like, 5′-ATGAAATTAT-TAAAAATATTGAGTT-3′ and 5′-TTATAAATAATGAAAAACACCC-3′). The results of these isolates were interpreted as false-positive. To analyse these false-positive results, the multiplex amplicons were cloned into pCR4-TOPO (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions and were sequenced with M13 primers.

3. Results and discussion

The number of intrinsic OXA identified in *Acinetobacter* spp. was recently expanded on the basis of whole-genome sequencing [12]. These represent a potential reservoir of carbapenem resistance genes in *Acinetobacter* spp. if overexpressed, as demonstrated in *A. bereziniae* (OXA-229) and *A. baumannii* (OXA-51-like) [1,6,10]. However, the present study focused on the distribution of acquired OXA in non-*baumannii Acinetobacter* spp., which appear to be the commonest carbapenem resistance determinants in this genus. Among a total of 453 *Acinetobacter* isolates, 23 were positive for an acquired OXA by multiplex PCR. The isolates were identified as *A. pittii* ($n = 18$), *A. nosocomialis* ($n = 2$), *Acinetobacter* junii ($n = 1$) and *Acinetobacter* genomic species 14TU/13BJ ($n = 2$). Of these, 9 isolates were resistant to both carbapenems tested, 1 was resistant only to meropenem and the remaining 13 isolates were carbapenem-susceptible (Tables 1 and 2).

Although isolate pairs BMBF436 and BMBF471, as well as AF263 and AF281, showed 98.8% and 96% similarity, respectively, by rep-PCR (data not shown), they originated from different countries and carried different OXA subclasses (Table 1). No epidemiological relatedness was found between the remaining isolates.

Sequencing confirmed the presence of four acquired OXA subclasses in 12 of the isolates. OXA-23 was identified in *A. pittii* ($n = 2$), *A. nosocomialis* ($n = 1$) and *Acinetobacter* genomic species 14TU/13BJ ($n = 1$). OXA-58 was identified in *A. pittii* ($n = 3$), *A. nosocomialis* ($n = 1$) and *A. junii* ($n = 1$). One *A. pittii* isolate co-harboured OXA-23 and OXA-58. Furthermore, two *A. pittii* isolates harboured OXA-255 (OXA-143-like) and OXA-72 (OXA-40-like), respectively. Identification of OXA-72 was based on a partial sequence (791 of 828 bp) (see Section 2.2). By Etest, eight isolates were resistant to imipenem and meropenem, three isolates were susceptible to both antimicrobials and one *A. junii* isolate was meropenem-resistant but imipenem-susceptible (Table 1).

IS elements were associated with bla_{OXA} in 10 of the 12 isolates (Table 1). IS*Aba3* flanked $bla_{OXA\text{-}58}$ in four carbapenem-resistant *Acinetobacter* isolates [*A. nosocomialis*, *A. pittii* ($n = 2$) and *A. junii*] and two carbapenem-susceptible *A. pittii* isolates (Table 1). Interestingly, sequencing 200 bp upstream from the start codon of $bla_{OXA\text{-}58}$ (including the putative promoter region) showed no difference between carbapenem-susceptible and carbapenem-resistant isolates. Consistent with the current results, IS*Aba3* associated with $bla_{OXA\text{-}58}$ has also been detected in carbapenem-susceptible *A. bereziniae* and *A. lwoffii* isolates [13]. Carbapenem resistance in the presence of IS-associated $bla_{OXA\text{-}58}$ might therefore, require additional strain-dependent mechanisms, for example, low permeability mediated by altered porin expression. IS*Aba1* was present upstream of $bla_{OXA\text{-}23}$ in all OXA-23-positive carbapenem-resistant isolates, but was absent downstream of the gene. *Eco*RV-digested and self-ligated plasmid DNA from *A. pittii* isolate S22 amplified an ca. 5 kb amplicon by inverse PCR. Sequencing of the $bla_{OXA\text{-}23}$ downstream region identified the first

Table 1
Characterisation of 12 *Acinetobacter* spp. isolates harbouring bla_{OXA} genes.

Isolate	Country of origin	Species	OXA multiplex PCR	Sequencing	IS element upstream of bla_{OXA}	Carbapenem MIC (mg/L)	
						IPM	MEM
AF281	Ireland	*A. pittii*	23-like	OXA-23	IS*Aba1*	>32	>32
BMBF436	Colombia	*A. pittii*	23-like	OXA-23	IS*Aba1*	>32	>32
S22	Singapore	*A. pittii*	23-like, 58-like	OXA-23, OXA-58	IS*Aba1*, IS*Aba3*	>32	>32
AF496	South Africa	*A. nosocomialis*	23-like	OXA-23	IS*Aba1*	>32	>32
BMBF461	South Korea	AGS 14TU/13BJ	23-like	OXA-23	IS*Aba1*	>32	>32
BMBF471	Italy	*A. pittii*	40-like	OXA-72	–	16	>32
AF626	Taiwan	*A. pittii*	58-like	OXA-58	IS*Aba3*	>32	>32
AF134	China	*A. pittii*	58-like	OXA-58	IS*Aba3*	1.5	3
AF263	Hong Kong	*A. pittii*	58-like	OXA-58	IS*Aba3*	1.5	2
G2-88b	Germany	*A. junii*	58-like	OXA-58	IS*Aba3*	1	16
AF614	Taiwan	*A. nosocomialis*	58-like	OXA-58	IS*Aba3*	>32	>32
AF726	USA (Indiana)	*A. pittii*	143-like	OXA-255	–	1	0.75

IS, insertion sequence; MIC, minimum inhibitory concentration; IPM, imipenem; MEM, meropenem; AGS, *Acinetobacter* genomic species.

Table 2
Characterisation of 11 *Acinetobacter* spp. isolates with false-positive bla_{OXA} PCR results.

Isolate	Country of origin	Species	OXA multiplex PCR	Closest similarity of sequenced multiplex PCR amplicons	Carbapenem MIC (mg/L)	
					IPM	MEM
G2-30	Germany	*A. pittii*	23-like	90% class C β-lactamase of *Acinetobacter oleivorans*	0.5	4
BMBF470	Italy	*A. pittii*	23-like	ND	0.25	2
G3-72	Germany	*A. pittii*	23-like	ND	0.5	4
G1-44	Germany	*A. pittii*	23-like	ND	0.25	2
AF874	USA (Utah)	*A. pittii*	58-like	Hypothetical proteins (cloning revealed three different sizes of inserts that were all sequenced and identified as different hypothetical proteins)	0.5	1
G3-6	Germany	*A. pittii*	58-like	ND	0.25	4
G3-11	Germany	*A. pittii*	58-like	ND	0.25	2
G1-56	Germany	*A. pittii*	58-like	ND	0.25	4
AF1	USA (Florida)	*A. pittii*	58-like	ND	0.5	2
AF257	Honduras	*A. pittii*	58-like	ND	0.75	4
BMBF460	South Korea	AGS 14TU/13BJ	23-like, 58-like	74% urea carboxylase of *Serratia proteamaculans* (70% carboxylase of *A. baumannii*)	>32	24

MIC, minimum inhibitory concentration; IPM, imipenem; MEM, meropenem; ND, not determined; AGS, *Acinetobacter* genomic species.

193 nucleotides of an ATPase gene that has recently been detected downstream of bla_{OXA-23}, e.g. as part of transposon Tn*6206*, in the *A. baumannii* BJAB0715 genome [14]. Sequences downstream of the bla_{OXA-23} genes in the remaining isolates showed 100% similarity to S22.

Although positive by multiplex PCR for an acquired bla_{OXA}, 11 mostly carbapenem-susceptible isolates did not amplify bla_{OXA} using other gene-specific primers in a singleplex PCR (see Section 2.3). This included 10 *A. pittii* isolates that were false-positive for OXA-23-like ($n = 4$) or OXA-58-like ($n = 6$) and one carbapenem-resistant *Acinetobacter* genomic species 14TU/13BJ isolate (BMBF460) that was false-positive for both genes (Table 2). Three isolates (BMBF460, G2-30 and AF874) representing species with an amplicon corresponding to each of the different OXA subclasses were selected for further investigation. Sequencing of cloned multiplex amplicons confirmed that the acquired bla_{OXA} was not present. BLAST search revealed the presence of three different hypothetical proteins in *A. pittii* isolate AF874, a putative novel class C β-lactamase in *A. pittii* isolate G2-30, and a urea carboxylase in *Acinetobacter* genomic species 14TU/13BJ isolate BMBF460 (Table 2). The mechanism of carbapenem resistance in BMBF460 remains to be determined. The presence of acquired bla_{OXA} in *Acinetobacter* spp. other than *A. baumannii* has been reported previously by multiplex PCR, however they are not always confirmed by sequencing [10,15]. The high rate of false-positive bla_{OXA} detected in this study (47.8%; 11/23 isolates) strongly suggests that confirmation of multiplex PCR results in non-*baumannii Acinetobacter* species is required by sequencing.

In summary, among 453 *Acinetobacter* isolates other than *A. baumannii*, acquired bla_{OXA} genes were identified in 12 *Acinetobacter* isolates mainly obtained from Europe and Asia, which correlates with previous reports. The first detection of acquired OXA in *Acinetobacter* spp. from South Africa and North America highlights their worldwide spread. In addition, this is the first report of OXA-23 in *Acinetobacter* genomic species 14TU/13BJ and of the coexistence of OXA-23 and OXA-58 in *A. pittii*. However, 11 isolates tested false-positive for bla_{OXA} using the established multiplex PCR. The high degree of false-positive results by OXA multiplex PCR in non-*baumannii Acinetobacter* spp. indicates that the results should be confirmed by additional methods, e.g. sequencing of the complete gene.

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Appendix 3. Other data relevant to the thesis

A3.1 Minimum inhibitory concentration of representative *Acinetobacter* spp.

Species	Country of origin	Minimum inhibitory concentration (µg/mL)									
		Erta-penem	Mero-penem	Ampi-cillin/ Sulbac-tam	Pipera-cillin/ Tazo-bactam	Cefe-pime	Ticarcillin/ Clavulanic acid	Genta-micin	Ami-kacin	Cipro-floxacin	Cefta-zidime
A. pittii T167	Thailand	>32	>32	4	>256	>256	>256	>256	>256	>32	>256
A. nosocomialis T228	Thailand	3	0.38	1	0.016	3	0.016	0.75	2	0.094	3
A. baumannii S36	Singapore	>32	12	4	>256	32	256	24	>256	>32	>256
A. baumannii T214	Thailand	4	0.5	1.5	0.016	3	12	1	3	>32	8
A. baumannii T229	Thailand	1	0.19	0.75	0.016	0.75	0.016	0.5	2	0.125	1.5
A. baumannii J133	Japan	1.5	0.5	0.75	0.016	>256	8	2	3	>32	>256
A. baumannii J2770	Japan	4	0.75	1.5	0.032	16	0.032	>256	>256	>32	192

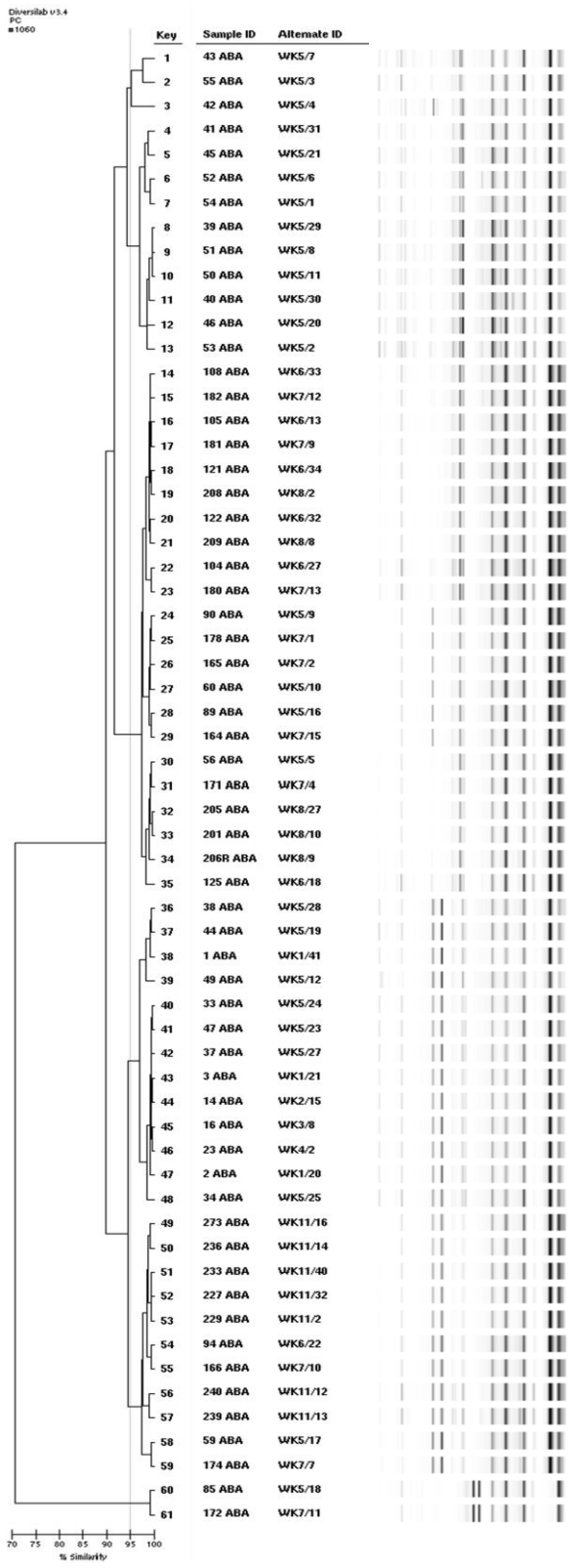
A3.2 Alignment of the OXA-51-like variants

A3.3 Molecular characterisation of *Acinetobacter baumannii* from Turkey

	ID	DVL Cluster	OXA-51	OXA-23	OXA-40	OXA-58	OXA-143	OXA-235	*gyrB* multiplex
52	1 ABA	3	1	1	0	0	0	0	*A. baumannii*
53	2 ABA	3	1	1	0	0	0	0	*A. baumannii*
54	3 ABA	3	1	1	0	0	0	0	*A. baumannii*
55	14 ABA	3	1	1	0	0	0	0	*A. baumannii*
56	16 ABA	3	1	1	0	0	0	0	*A. baumannii*
57	23 ABA	3	1	1	0	0	0	0	*A. baumannii*
58	33 ABA	3	1	1	0	0	0	0	*A. baumannii*
59	34 ABA	3	1	1	0	0	0	0	*A. baumannii*
60	37 ABA	3	1	1	0	0	0	0	*A. baumannii*
61	38 ABA	3	1	1	0	0	0	0	*A. baumannii*
62	39 ABA	1	1	1	0	0	0	0	*A. baumannii*
63	40 ABA	1	1	1	0	0	0	0	*A. baumannii*
64	41 ABA	1	1	1	0	0	0	0	*A. baumannii*
65	42 ABA	1	1	1	0	0	0	0	*A. baumannii*
66	43 ABA	1	1	1	0	0	0	0	*A. baumannii*
67	44 ABA	3	1	1	0	0	0	0	*A. baumannii*
68	45 ABA	1	1	1	0	0	0	0	*A. baumannii*
69	46 ABA	1	1	1	0	0	0	0	*A. baumannii*
70	47 ABA	3	1	1	0	0	0	0	*A. baumannii*
71	49 ABA	3	1	1	0	0	0	0	*A. baumannii*
72	50 ABA	1	1	1	0	0	0	0	*A. baumannii*
73	51 ABA	1	1	1	0	0	0	0	*A. baumannii*
74	52 ABA	1	1	1	0	0	0	0	*A. baumannii*
75	53 ABA	1	1	1	0	0	0	0	*A. baumannii*
76	54 ABA	1	1	1	0	0	0	0	*A. baumannii*
77	55 ABA	1	1	1	0	0	0	0	*A. baumannii*
78	56 ABA	2	1	1	0	0	0	0	*A. baumannii*
79	85 ABA	4	1	1	0	0	0	0	*A. baumannii*
80	59 ABA	3	1	1	0	0	0	0	*A. baumannii*
81	89 ABA	2	1	1	0	0	0	0	*A. baumannii*
82	90 ABA	2	1	1	0	0	0	0	*A. baumannii*
83	60 ABA	2	1	1	0	0	0	0	*A. baumannii*
84	94 ABA	3	1	1	0	0	0	0	*A. baumannii*
85	104 ABA	2	1	1	0	0	0	0	*A. baumannii*
86	105 ABA	2	1	1	0	0	0	0	*A. baumannii*
87	108 ABA	2	1	1	0	0	0	0	*A. baumannii*
88	excluded								
89	121 ABA	2	1	1	0	0	0	0	*A. baumannii*
90	122 ABA	2	1	1	0	0	0	0	*A. baumannii*
91	125 ABA	2	1	1	0	0	0	0	*A. baumannii*
92	171 ABA	2	1	1	0	0	0	0	*A. baumannii*
93	172 ABA	4	1	1	0	0	0	0	*A. baumannii*
94	174 ABA	3	1	1	0	0	0	0	*A. baumannii*
95	164 ABA	2	1	1	0	0	0	0	*A. baumannii*
96	165 ABA	2	1	1	0	0	0	0	*A. baumannii*
97	178 ABA	2	1	1	0	0	0	0	*A. baumannii*
98	166 ABA	3	1	1	0	0	0	0	*A. baumannii*
99	180 ABA	2	1	1	0	0	0	0	*A. baumannii*
100	181 ABA	2	1	1	0	0	0	0	*A. baumannii*
101	182 ABA	2	1	1	0	0	0	0	*A. baumannii*
102	208 ABA	2	1	1	0	0	0	0	*A. baumannii*
103	209 ABA	2	1	1	0	0	0	0	*A. baumannii* not confirmed as
104	206 ABA	2	1	1	0	0	0	0	*A. baumannii*
105	201 ABA	2	1	1	0	0	0	0	*A. baumannii*
106	205 ABA	2	1	1	0	0	0	0	*A. baumannii*
107	227 ABA	3	1	1	0	0	0	0	*A. baumannii*

108	229 ABA	3	1	1	0	0	0	0	*A. baumannii*
109	233 ABA	3	1	1	0	0	0	0	*A. baumannii*
110	236 ABA	3	1	1	0	0	0	0	*A. baumannii*
111	239 ABA	3	1	1	0	0	0	0	*A. baumannii*
112	240 ABA	3	1	1	0	0	0	0	*A. baumannii*
113	273 ABA	3	1	1	0	0	0	0	*A. baumannii*

A3.4 Dendrogram of 61 *Acinetobacter baumannii* isolates from Turkey



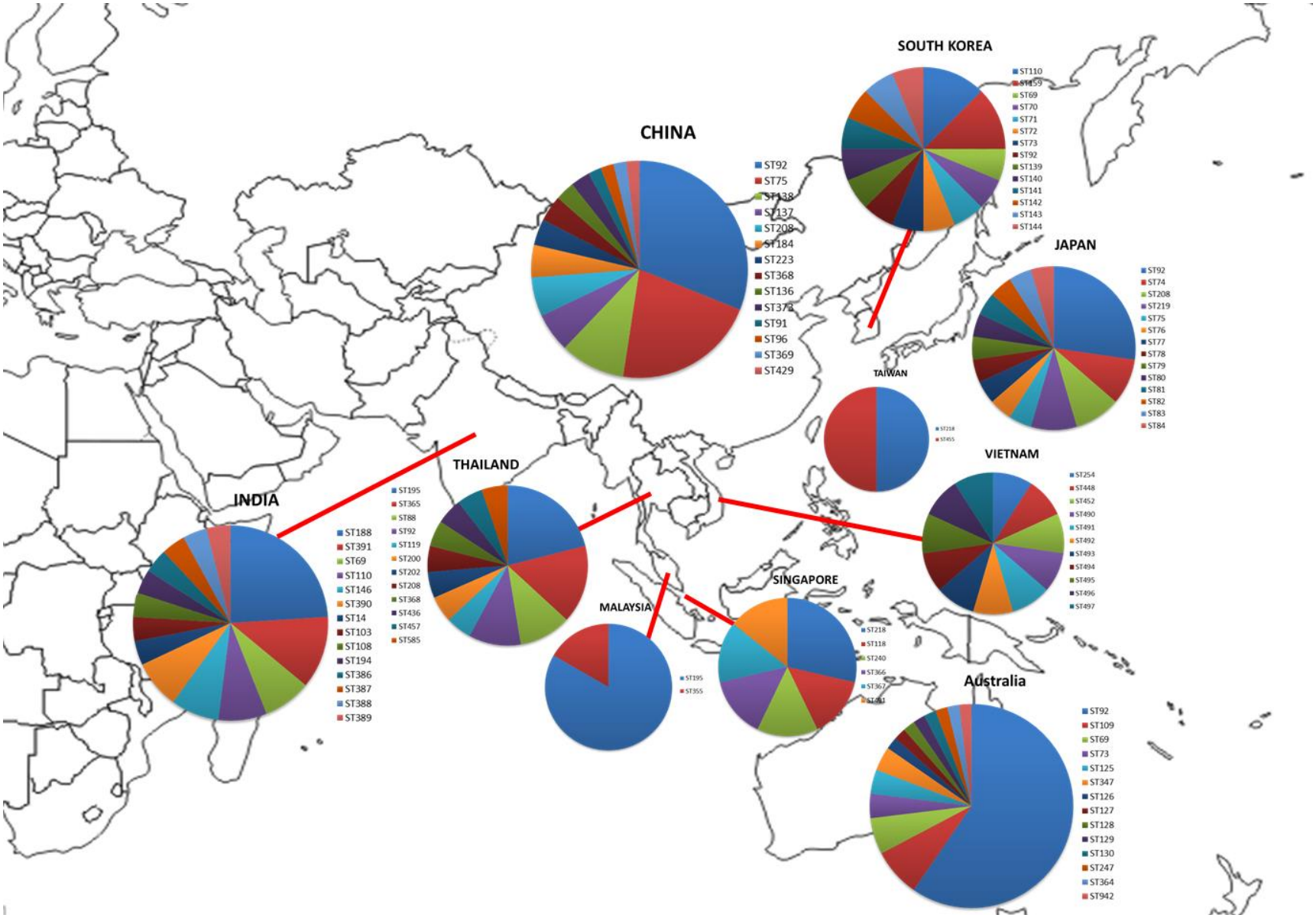
A3.5 List of primers used to solve the ambiguity of the order of scaffolds of *Acinetobacter* spp. genome and other primers for molecular characterisation

Primer name	Primer sequences	Purpose
T87_1F	AGTAATTTCACCCGGTTTAGGG	Closing genome gaps
T87_1R	TTGGAAATACTGGGTTTCTTCG	
T87_2F	GCGATAGTGAACGGATTGAG	
T87_2R	GCTCGGACACCTGAATTAGC	
T87_3F	TGCTTCCAGATGTATGCTCTTC	
T87_3R	GGAGAAACTGTCCGAGGTTATG	
T87_4F	AGGTGGAGCTGACTTCATCC	
T87_4R	GTATCCTTGCCGATCACGAC	
T87_5F	TCAACGCCTGCAATAATGG	
T87_5R	TCATGAGCTTTGGCACAGG	
T87_6F	ATGCTGAACCGTACAACCAG	
T87_6R	GGTGTAGTCGCTTGTGTGTTG	
T87_walk_F	GTTCGCCGGATAAGTAATTTGC	
T87_walk_R	ATGGTTGCATTCGGTAAGCAC	
J65gap2_1	CCGAGCATCCGTATGAGACT	
J65Gap2_2	TGTCCGAGGTTATGTTGACG	
Sup_F	CCCCATATCACCGACAGTTC	
Sup_R	TGAAATGGGTTCTCCTCTGG	
DOWNOXA_1	CGTTCCTCTAACTTTCCTG	
DOWNOXA_2	TGTCCTGATACTCATCCTGTC	
UPOXA_1	CACCAGCAACTATCACTGC	
UPOXA_2	GAATAACCAGCACACCTGAG	
Ac696F	TAYCGYAAAGAYTTGAAAGAAG	*rpoB*
Ac1093R	CMACACCYTTGTTMCCRTGA	
Ac1055F	GTGATAARATGGCBGGTCGT	
Ac1598R	CGBGCRTGCATYTTGTCRT	

Sp2F	GTTCCTGATCCGAAATTCTCG	Multiplex PCR for *gyrB*
Sp4F	CACGCCGTAAGAGTGCATTA	
Sp4R	AACGGAGCTTGTCAGGGTTA	
D14	GACAACAGTTATAAGGTTTCAGGTG	
D19	CCGCTATCTGTATCCGCAGTA	
D16	GATAACAGCTATAAAGTTTCAGGTGGT	
D8	CAAAAACGTACAGTTGTACCACTGC	
G1_csuEF	CTTTAGCAAACATGACCTACC	SG typing
G1_csuER	TACACCCGGGTTAATCGT	
G1_ompAF	GATGGCGTAAATCGTGGTA	
G1_OXA66F	GCGCTTCAAAATCTGATGTA	
G1_OXA66R	GCGTATATTTTGTTTCCATTC	
G1and2_ompAR	CAACTTTAGCGATTTCTGG	
G2_csuEF	GGCGAACATGACCTATTT	
G2_csuER	CTTCATGGCTCGTTGGTT	
G2_ompAF	GACCTTTCTTATCACAACGA	
G2_OXA69F	CATCAAGGTCAAACTCAA	
G2_OXA69R	TAGCCTTTTTTCCCCATC	
AB_70F_RPOD	ACGACTGACCCGGTACGCATGTAYAT GMGNGARATGGGNACNGT	MLST
AB_70FS	ACGACTGACCCGGTACGCATGTA	
AB_70R_RPOD	ATAGAAATAACCAGACGTAAGTTNGC YTCNACCATYTCYTTYTT	
AB_70RS	ATAGAAATAACCAGACGTAAGTT	
AB_CPN_3F2	ACTGTACTTGCTCAAGC	
AB_CPN_R2	TTCAGCGATGATAAGAAGTGG	
AB_GDH_SEC_F	ACCACATGCTTTGTTATG	
AB_GDH_SEC_R	GTTGGCGTATGTTGTGC	
AB_GDHB_1F	GCTACTTTTATGCAACAGAGCC	

AB_GDHB_775R	GTTGAGTTGGCGTATGTTGTGC	
AB_gltA_Citrato_F1	AATTTACAGTGGCACATTAGGTCCC	
AB_gltA_Citrato_R12	GCAGAGATACCAGCAGAGATACACG	
AB_GPI_F1	AATACCGTGGTGCTACGGG	
AB_GPI_R1	AACTTGATTTTCAGGAGC	
AB_GYR_M13_F	CAGGAAACAGCTATGACC	
AB_GYR_UP1ER	CAGGAAACAGCTATGACCAYGSNGGN GGNAARTTYRA	
AB_gyrB_APRU_F	TGTAAAACGACGGCCAGTGCNGGRTC YTTYTCYTGRCA	
AB_gyrB_M13_-21	TGTAAAACGACGGCCAGT	
AB_REC_RA1	CCTGAATCTTCYGGTAAAAC	
AB_REC_RA2	GTTTCTGGGCTGCCAAACATTAC	

A3.6 Figure demonstrates STs available from MLST database (Oxford scheme) from Asia and Oceania.



A3.7 Table of epidemiology studies used in Chapter 1

Australia

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	177 *Acinetobacter* spp.	1995-2000	WM, Sydney	PFGE	8 pulsotypes were identified	CR isolates appeared in 1999 (different pulsotypes from CS)	*A. pittii* (pulsotype III) *A. nosocomialis* (pulsotype IV)	OXA-23 in all resistant isolates (13) and was found in different pulsotypes	Valenzuela JK, 2007
	3 *Acinetobacter* spp.		PW, Sydney		2 pulsotypes				
	6 *Acinetobacte*r spp.		RB, Brisbane		2 pulsotypes				
2	32 MDR AB	2002-2007	Sydney	REP-PCR, genotypes	2 REP-PCR types, 3 clonal groups	REP-PCR can't discriminate 6 isolates that have integron	-	OXA-23 in all CR	Mak JK, 2009
3	53 MDR AB	2001-2003	RBWH, Brisbane	PFGE, PAR	3 Common pulsotypes, 8 Single	One major clone: majority of AB from RBWH since 2000			Long YB, 2009
4	~41 patients CRAB	2006	RBWH, Brisbane	PFGE (4)	Identical	Successful control: cleaning w Virkon			Doidge M, 2010
5	33 MDR AB (27 CR)	1998-2008	RBWH, Brisbane	PFGE, DiversiLab, MLST	4 clones 6 STs	ST92 (22) predominant (2000-2008), ST69 (3), 73 (2), 125 (2),126 (1), 127(1)		OXA-23 in all CR	Runnegar N, 2010
6	13 MDR AB (10 from #ref 2)	1997-2007	Sydney	SBT, *recA*, OXA-51-like	IC1 (6), IC2 (7)				Post V, 2010
7	61 CRAB	2000-2010	Sydney, Brisbane, Canberra,	SBT, *recA*, OXA-51-like, REP-PCR, RAPD	IC2	Study Aminoglycoside resistant in IC2		OXA-23 all	Nigro SJ, 2011
8	90 AB	2001-2010	Sydney, Brisbane, Canberra, Melbourne	SBT, *recA*, OXA-51-like, MLST	IC1 (11), IC2 (52)	ST109 (3), ST247(1)		OXA-23 (3), OXA-58 (2)	Hamidian M, 2011

China

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1		2000-2009	Nationwide			*Acinetobacte*r: nosocomial pathogen			Yong-Hong Xiao, 2011

						8.8% in 2000 and 15.5% in 2009			
						IMP/MER resistance rate: 2.5/4.1% in 2000 and 50/52.4% in 2009			
						85% is A. baumannii			Ni, 2007 Sun, 2009a
		2003-2008				MER: <10% in 2000 and >50% in 2010		OXA-23, 58, 66?	Wang, 2010b
								OXA-23	Zhou, 2007
			11 teaching hospitals			Clonal spread in 11 hospital		97.7% OXA-23 IMP-8 (1) OXA-66 (some)	Wang, 2007
			16 cities		CC22 closely related to IC2	86.8% CRAB 45.9% CSAB		Almost all OXA-23	Fu, 2010
2	221 IMP-resistant *Acinetobacter* spp. AB (187)	1999-2005	11 teaching hospitals	PFGE	15 types	Clone P Clone A Most prevalent	GS10/11 (18) A. pittii (2) A. nosocomialis (5)	OXA-23 (97.7%) OXA-58 (7) OXA-72 (1) IMP-8 (1); nonAB OXA-66 (18 representative isolates)	Wang H, 2007
3	37 IMP-resistant AB	2007	Shenyang	PFGE, MLST	4 types	2 epidemic clones ST92 (18) ST75 (17) Both are CC92		OXA-23 (34) OXA-58 (24) PER-1 (35) AdeABC important for high IMP-resistance	Zhang, 2010
4	49 MDRAB	2007	3 military hospitals	DiversiLab	5 major clones	2 major clones were widespread in 3 hospitals CC92?		OXA-23 (73.5%) OXA-58 (2%) ISAba1/OXA-51 (12.2%)	
5	2109 AB	2009-2010	18 provinces	PFGE	Diverse	Different plasmid		NDM-1 in 4 isolates	Chen, 2011
6	57 CRAB 20 CSAB	2007-2009	Chongqing	PFGE, MLST	6 pulsotypes 5 STs	2 clones in CRAB ST75 (49%) ST137 (51%) Both: CC92		OXA-23 (100%)	
7	174 MDRAB 86 CRAB	2009 (Jan-June)	Hunan	17 (1	4 types	Clonal spread		OXA-23 (90.7%)	Chen, 2011
8	46 CRAB 19 PSAB	2004-2009	Beijing	REP-PCR, MLST	8 types 23 STs	ST92 (24), ST191 (7), ST75 (5), ST118 (2), STn17(1) All are CC92		OXA-23 (80.4%)	Huang, 2013

9	95 IMP-R AB	2003-2004	University Hospital	PFGE	6 clonal patterns	2 predominant pattern A (50), B (35)		All OXA-23 and OXA-66 (51-like)	Han Zhou, 2007
10	76 CRAB, MDRAB	2008-2011	ICUs	DiversiLab (24 hospital infections)	4 groups	2 major groups: epidemic status of nosocomial infection caused by CRAB		All OXA-23	Wei Jiang, 2010

Japan

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-*baumannii*	Resistance gene	Reference
1	88 *Acinetobacter* spp. 3 CRAB *CR rate 1998-2002	1998-2002	22 centres			CR rate 5.9% in 1998 and 4.8% in 2002		IMP-1	Jones RN, 2004
2	902 *Acinetobacter* spp.	2004	100 centres			CR rate 3.2%			Ishii,
3	201 ABC	2007-2009	Nagasaki			CR 1 isolate (patient died from pneumonia) Mortality rate 24.3%			
4	598 *Acinetobacter* 27 carb-non-susceptible 14 AB 13 non-*baumannii*	2007	72 hospitals (Nationwide)	PFGE, MLST	3 types (2 are CC92) 3 STs	4.5% carb-non-susceptible *Acinetobacter* spp. ST208 (10) , ST219 (3) are CC92 ST222 (1) is CC276	Found in diverse area (13) *A. pittii* (3): OXA-58 (3), IMP-1 (2) *A. nosocomialis* (4): IMP-1 (3), IMP-2 (2) *A. calco* (4), IMP-1 (4) *A. lwoffii* (2): OXA-58 (1), IMP-1 (2)	ST222 isolate: IMP-1, OXA-58 OXA-23 (6) ISAba1/OXA-51 (12)	Kouyama Y, 2012
5	305 *Acinetobacter* spp. 55 Carb-non-susceptible ACB 52 AB 3 *A. pittii* 18% CNSACB	2009-2010	176 centres	MLST	6 STs ST92, 106, 74, 79 are CC92	ST92 (76.9%) ST106 (9.6%) ST74 (3.8%) ST76 (3.8%) ST188 (3.8%) ST195 (1.9%): OXA-23 producing	OXA-66 (82.7%), OXA-80 (9.6%), OXA-83 (1.9%), OXA-51 (1.9%) OXA-23 (1.9%) ISAba1/OXA-51 (69%)	*A. pittii*: IMP-19 (1)	Endo S, 2012

6	48 *Acinetobacter* spp. with MBL genes	2001-2006	5 hospitals	PFGE, MLST (Pasteur)	*A. pittii* ST119 (w IMP-19) is predominant	*A. baumannii* (1): ST120 (not related to IC1,2 or 3) A. pittii (25): ST119 (18), ST63, 64, 93, 121 *A. nosocomialis* (5): ST68, 71 *A. bereziniae* (9) Unclassified (3) Others (5)	*A. baumannii* IMP-19	*A. pittii* IMP-19 (23) IMP-1 (1) IMP-11 (1) OXA-58 (2) *A. nosocomialis* IMP-19 (4) *A. bereziniae* IMP-19 (9)	Ichiyama S, 2013
7	1 *A. baumannii* Transferred from India to Japan	2011	Indian hospital Jikei Uni Hospital, Tokyo	MLST	ST222 (missing in pubmlst)	Not CC92		OXA-51 (no IS*Aba1*) OXA-23 NDM-1	Nakazawa Y, 2013

Hong Kong

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	60 clinical 80% *A. baumannii*	1990-1994	Queen Mary	AP-PCR	Unrelated strains	7.4% of all bacteria 5.8% resistance to IMP (stable)			Siau, 1996
2	18 *Acinetobacter* spp. bacteremia	1995	Queen Mary	AP-PCR		16 *A. baumannii* 2. *A. lwoffii*		All susceptible to IMP	Siau, 1999
3	45 MDR AB 24 CRAB	2005-2006	5 hospitals	PFGE, MLST	2 types 2 STs	ST26 (24) in CRAB (non-CC92) ST22 (19) CSAB		OXA-23 all CRAB	Ho, 2010

India/Pakistan

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	20 *Acinetobacter* spp. 17 *A. baumannii* (11 CRAB) 1 *A. pittii* 1 *A. nosocomialis* 1 unidentified	2008	2 hospitals	PFGE	Not related	3 groups: >85% have same OXA-51-like Interhospital spread		OXA-23 all CR OXA-66, 68, 69, 98, 99, 114, 65 variant, 100	Evans BA, 2010
2	132 *A. baumannii*	2010-2011	Panjab, India			42 (31.8%) CR 4 PDR: tiger and colistin			Mahajan G, 2011

3	88 *A. baumannii*	2011-2012	Tertiary hospital, India			10 (11%) IMP resistance 12 () MEM resistance			Ghakraborty B, 2011
4	94 patients 20% community-acquired *Acinetobacter* infection	2009	Tertiary hospital, northern India			70% MDR 70% crude mortality rate 76% CRAB (MER) 13% colistin R			Mathai AS, 2011
5	4180 samples from ICU 4.5% *Acinetobacter* spp.	2010-2011 (2 yrs)	Kolkata, India			5.2% IMP-R 9.8% MEM-R	74% ACB 14.2% *A. lwoffii* 7.8% *A. haemolyticus* 3.8% *A. junii*		Rit K, 2012
6	23 *A. baumannii*	2011	2 hospitals, Delhi, India	MLST	ST	ST110 (2) ST188 (3) ST146 (2) ST69 (2) ST103 (1) ST108 (1) ST194 (1)	Novol ST ST368 (1) ST387 (1) ST388 (1) ST389 (1) ST390 (3) ST391 (3) 1-10-10-6-1-4-14		Rynga D, 2013
7	140 *Acinetobacter* spp.	2010-2011	Tertiary hospital, North India			20% MEM-R (28)		IMP-1 (8) IMP-2 (0) VIM-1 (2) VIM-2 (1) Efflux (4)	Sinha N, 2013
8	55 IMP-R *A. baumannii*	2007 (4 mo)	Tertiary hospital, South India	RAPD	6 clusters	2 major cluster (17, 13)		IMP-1 (42%) No VIM-2	Karthika RU, 2009
9	74 *A. baumannii*	2011 (5 mo)	University Hospital, Varanasi	ERIC-PCR	3 NDM: different patterns	30 MBL activity:		3 NDM 5 VIM	Mishra S, 2013
10	30 IMP-R AB	2008-2009	Tertiary hospital, Delhi			All susc to colistin Using real-time PCR		All IMP-1, VIM-1 IMP-2 (15) VIM-2 (6) OXA-23 (14) NDM-1 (9/14)	Niranjan DK, 2013
11	156 *Acinetobacter* spp.	1997-2006	ICUs at King Edward Memorial Hospital			93% were B lactamases + 11.5% (18) positive: ESBL DDST		10 PCR for ESBLs 5 PER-1 (chromosomal)	Litake GM, 2009

12	62 *Acinetobacter* spp. (48 AB, 14 non-AB)	2007-2008 (6 mo)	4 hospitals in south India	RAPD-PCR	40 types	Nonclonal spread	OXA-23 (28.5% OXA-24 (64.3%) OXA-58 (35.7%)	OXA-23 (47.9%) OXA-24 (22.9%) OXA-58 (4.2%)	Karunasagar A, 2011

Malaysia

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-*baumannii*	Resistance gene	Reference
1	109 *Acinetobacter* spp.	1987, 1996-1998	UMMC, KL	REP-PCR	4 types	2 major types (92% of isolates) were persist over a decade in this hospital	1987(21) 4.8% IMP-R 1996-1998 (88) 36.4% IMP-R		Misbah S, 2004
2	23 nosocomial blood stream pathogens	2003-2007	3 ICUs			22% *Acinetobacter* spp. (5)			Gopal Katherason S, 2010
3	185 *A. baumannii* 170 (92%) clinical 15 (8%) environment and hands of HCW	2006-2009	Teaching hospital ICU	PFGE	8 types (70% similarity)	Clinical isolates 96.5% IMP-R, 98.2% MEM-R All colistin S	A persistent clone of MDR-AB Isolates form hands of HCW were closely related to clinical isolates suggesting transmission form HCWS to patients by hands		Boon Hong Kong, 2011

Singapore

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	60 *Acinetobacter* spp.	1998-1999	4 medical centres, Malaysia 2 sites, Singapore			13.3% IMP-R			Biedenbach DJ, 1999

2	*Acinetobacter* spp. 41 ACB IMP-R (53%)	2004 (8 month)	Tertiary care hospital	PCR-based fingerprint	No dominate clone (but no screening for colonization)	4 small clonal groups for 50% of isolate 52% IMP-R in 2004 and constant 2002-2006			Tan TY, 2007
3	171 *Acinetobacter* spp. 81.3% *A. baumannii* (139)	2006-2007	5 hospitals			*A. baumannii* 70.5% carb-R 0.7% colistin R 7.9% tigercycline R	25% carb-R		Tan TY, 2008
4						Over all 49.6% of Acinetobacter: Carb-R in 2007			Hsu LY, 2007
5	1990 MDRAB emerged in the burns unit of SGH 1992: 2 isolates colistin R						2 *A. pittii* and 1 *A. nosocomialis*: OXA-58, IMP-4 (from Brown S, 2005)		Koh TH, 2008
6	47 patients 82 strain of *A. baumannii*	2001-2006				A. baumannii (33%): most common causing nosocomial infections 78.7% IMP-R			Chin H, 2007
7	2000 *Acinetobacter* spp.	2008-2008	4 public hospitals			46.7% *Acinetobacter* IMP-R 50% blood IMP-R	Possible association between prescription of carbapenems and IMP-R Acinetobacter		Hsu LY, 2010
8	193 ACB 78.7% *A. baumannii* 9.3% *A. pittii* 11.9% *A. nosocomialis*	2006 (1 mo)	6 hospitals	AFLP MLST (pasture)		IC1: outbreak in 2001 (OXA-69) IC2: outbreak in 2001 and 2006 (OXA-66) ST25, ST111 unrelated to IC1, 2, or 3	*A. baumannii* 72.4% CR *A. pittii* 38.9% *A. nosocomialis* 34.8%	OXA-23 in *A. baumannii* (108), A. noso (8) IS*Aba1*/OXA-51 in IMP-R AB (12) OXA-58 in *A. baumannii* (1) *A. nosocomialis* (4) *A. pittii* (8)	KOH TH, 2011
9	114 IMP-R ACB	5 mo each of 1996 2001	Tertiary general hospital	PFGE	16 pulsotypes A (36.3% for carb producing isolates)	IMP-R 1996 (7.7%) 2001 (21.2%)	3 *A. pittii* OXA-23 (1) OXA-58, IMP-4 (2)	OXA-23 (37) OXA-96 (58-like) (1) OXA-51-like	Tse Hsien Koh, 2007 Tse Hsien Koh, 2007

							2 *A. nosocomialis* OXA-58, IMP-4 (2)	-64 (13), -66 (14), -69 (4), -88 (3), -95, -91, 93	

Korea

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1.	142,107 and 68,391 6.6% *Acinetobacter* spp.	2009	24 hospitals and 2 Com Labs			IMP-R 1% 1997 22% 2007 51% 2009			Lee K, 2011 KONSAR group
2	547 *Acinetobacter* spp. 388 AB (272 CNSAB) *A. nosocomialis* (82) *A. pittii* (62) *A. bereziniae* (13) A. GS 14TU (2)	2008	19 hospitals	DiversiLab, MLST	57 band patterns 57 STs	70% CNSAB All CNSAB: 3 band patterns (similar to IC2 by Higgins) 5 STs of CNSAB ST92 (159), ST75 (64), ST137 (3), ST138 (38), are CC92 ST69 (8) DLV with ST92 *A. baumannii* (1) and *A. bereziniae* (1) low level resistance to colistin, A. GS 14TU (2): high level	IMP-1 (6) VIM-2 (4) OXA-23 (1 *A. pittii*)	OXA-23 (169/272 CNSAB) ISAba1/OXA-51 (89O	Lee Yangsoon, 2011
2.1	IMP-non-susceptible *Acinetobacter* spp. *A. baumannii* (44) *A. pittii* (10) *A. nosocomialis* (2)	2008-2010	Uni Hospital, Daejeon	Rep-PCR	2 types of AB Diverse band pattern : *A. pittii*, *A. nosocomialis*		*A. pittii* IMP-1 (10) 100% *A. nosocomialis* VIM-2 (2) 100%	*A. baumannii* OXA-23 (100%) IS*Aba1*/OXA-51 (88.6%)	Ji Youn Sung, 2011
3	36 CRAB	2004-2006	17 non-tertiary hospitals	DiversiLab, MLST	8 rep-PCR patterns 2 STs	ST69 (17) 47.2% ST92 (19) 52.8%)	These 2 clones: nationwide and both tertiary and non-tertiary hospitals	IS*Aba1*/OXA-51 (25) 69.4% IS*Aba1*/OXA-23 (14) 38.9%	Sunok Park, 2013

4	287 *Acinetobacter* spp. from blood stream infection *A. baumannii* (44.2%) *A. nosocomialis* (34.1%) *Acinetobacter* close to 13TU (9.8%) *A. pittii* (5.2%) *A. calcoaceticus* (1%) Non-ACB (5.6%) 65 CRAB isolates	2003-2010	one hospital	MLST	9 STs CC92: ST92 ST75 ST138 ST220 DVL of CC92 ST69, 70, 71 ST110 ST20	ST92 (47.8%), ST69(15.9%): most prevalent clones in 2003-2007 ST75 (39.7%), ST138 (25.9%) had increased in 2008-2010 suggesting substitution of main clones (still in CC92) into more resistant clones	IMP/MEM-R 2003-2007: 12.9%-20.5% 2008: 41.4/41.5% 2008-2012 polymyxin-R in few isolates High colistin-R in *A. nosocomialis* (43.9%) ST75, ST138 showed high carb-R, ST92 showed moderate resistance	All ST75, 138 (and one ST92) had OXA-23 ST92 (66.7%), ST69 (66.7%): high prevalent of PER OXA-23 (38) 58.5% IMP-1 *A. baumannii* (3) *A. pittii* (7)	Young Kyoung Park, 2012
5	44 *of A. baumannii* IC2	2003-2010	Multiple hospitals	MLST	Both CR, CS ST92 (19) ST75 (7) ST138 (5) ST69 (9) ST71 (2) ST220 (1)	Most CRAB in Korea have originated from the same ancestor with a globally disseminated clone (IC2)		ST75 AbaR4-type with Tn2006 ST138; Tn2006 not in AbaR	Dae Hun Kim, 2012
6	2 Carb-R *A. nosocomialis*		Korea, Thailand			Hypothesised that AbaR-4 in *A. nosocomialis* have been transferred from *A. baumannii* IC2		OXA-23 located in AbaR4-type	Dae Hun Kim, 2012
7	29 MDR AB 22 CRAB 7 CSAB	2007-2011	Single university hospital, Daejeon	SBT, rep-PCR, MLST	IC2 (27): OXA-66 IC1 (2): OXA-69 2 rep-PCR patterns: correlate with IC from SBT	MLST of IC2 5STs: ST75, 92, 137, 138, 357 MLST of IC1: ST109		AbaR7 found in IC1 (2) isolates OXA-23 (11/22) *armA* (24/29)	Ji Youn Sung, 2012
8	253 *A. baumannii* (93.7%) *A. nosocomialis* (10) *A. pittii* (5)	2008-2009	73 hospitals, 10 Asian countries	MLST (108 isolates tested)	36 STs	76.9% CC92 (83) from all 8 countries: 18 STs ST92 (19) (IN, MY, PH, TW, TH), ST195		82.5% CRAB (208) 108 tested: ISAba1/OXA-51 6.5 % (7)	

	> 60% *A. baumannii* causing HAP were resistant to all antimicrobial agents except the polymyxins indicting, which indicates that the continuing increase of highly MDR A. baumannii in Asia (Chung DR, 2011)					(18) (TH, MY) are predominant CC108: prevalent in India (5) CC119 (3), CC254 (3), singleton (11)		OXA-23 94.4% (102) OXA-24 6.5% (7) all from Taiwan All CC92 (47 tested) : AbaR4-like (AbaR25-I most prevalent (34.8%))	
9	158 A. *baumannii*	2011	13 hospitals: Nationwide				Resistance rate (IMP/MEM) 2006-2007 20/23.3 2008 25.4/28.8 2011 55.4/37.5		Kyungmin Huh, 2013
10	31 amikacin and cipro-R *A. baumannii* 30 *A. baumannii* 1 *A. calcoaceticus*	2010 (4 mo)	Single hospital	ERIC-PCR, MLST	4 STs	CC118: ST191 (28) ST208 CC110: ST229 ST207 (1 A. calco)	30/31 OXA-23 28/31 armA All ORDR mutations (both gyrA, parC)		Seung Bok Hong, 2013
11	69 CRAB 5 CRAB (2005)	2009-2010	Single hospital, Daegu	PFGE, SBT, MLST (both Pasture, and Oxford)	9 pulsotypes ST2 all; Pasture	2 main pulsotypes: 63 SG1 (IC2) isolates: MLST Oxford ST191 (56) ST208 (6) ST353(1)	5 CRAB (2005) showed ST353 suggesting that isolates form 2005 were not clonally related to 2009-2010	All OXA-23 PER (9) *armA* (58/69) 84%	Mamata Gurang, 2013
12	8 MDR AB	2001-2012 (4mo)	NICU, Daejeon	SBT, MLST, rep-PCR	1 clone ST357 (IC2)	All ST357; have not reported in Korea before	All CS but resistant to amikacin, genta, 4th gen cep and cipro	All has armA	Ji Youn Sung, 2013
13	52 IMP-nonsusceptible *A. baumannii*	2007-2011	University hospital, Daejeon	SBT, MLST	6 STs All CC92, IC2	ST69 (2), ST92 (5), ST75 (2), ST137 (16), ST138 (25), ST358 (1)		OXA-23 (31) IS*Aba1*/OXA-51 (23) *armA* (45)	Hye Hyun Cho, 2013
14	30 XDR All IMP-R	2007 (4 mo)	7 tertiary hospitals	PFGE	28 types	Both clonal and non-clonal dissemination No interhospital transmission		OXA-23 (23) 77% all with IS*Aba1* IS*Aba1*/OXA-51 (7) 23%	Yoon Soo Park, 2010

15	35 MDR	2007-2008	3 Uni Hospitals	Rep-PCR	Hospital A, C diverse, B has 2 patterns	2 predominate clones		OXA-23 (22.9%) with high MIC IS*Aba1*/OXA-51 (60%) IMP-1 (8.6%)	Sun Hoe Koo, 2010
16	28 IMP-R AB Clinical isolates 7 IMP-R AB environmental isolates	2004	2 ICUs, Uni Hospital	PFGE	One major clone; 82% of clinical and 86% of environmental			IS*Aba1*/OXA-51 (93.7%) IMP-1 (n=2) (6.3%)	Chaulagain BP, 2012
17	190 IMP-NS *Acinetobacter* spp. 92.2% (178) *A. baumannii*	2007 (3 mo)	12 tertiary-care hospitals	PFGE (140 isolates of OXA-23) Sequence type group (37 rep isolates)	15 types 37 subtypes	89% of *A. baumannii* with OXA-23 had identical PFGE pattern 76% was IC2 11/12 OXA-182 *A. baumannii* had the same PFGE pattern	12 non-*baumannii* IMP-1 (75%) VIM-2 (17%) SIM-1 (8%)	OXA-23 (80%) IS*Aba1*/OXA-51 (12%) Novel OXA-182; 93% to OXA-143 (7%) VIM-2 (1%)	Chang-Ki Kim, 2010
18	86 *A. baumannii*	2007-2010	A hospital, Gangwon province	AFLP Rep-PCR	All except one (85) showed identical AFLP profiles and similar band patterns (rep-PCR)	Clonal spread		All OXA-23 31 TEM 32 PER-1 *armA* (81%)	

Taiwan

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	614 ACB 120 (19.5%) MDR ACB	2006 (a year)	Hsin-Chu general hospital	PFGE	26 types 36 subtypes	Many diff MDR clones found in this hospital			Ming-Feng Lin, 2009
2	167 *A. baumannii* (hospitalised in ICU)	2005	10 major teaching hospitals, Nationwide	PFGE (25 XDR isolates)	6 pulsotypes One or more of 6 clones were present in all of the diff major Taiwanese hospitals	IMP-R AB 25% in 2005 22% in 2000 6% colistin R (2005) Supporting the endemic propensity of *A. baumannii*			Shio-Shin Jean, 2009
3.	94 *A. baumannii* 73 from patients 31 from environment	2006 (a year)	3 ICUs in university	Rep-PCR, RAPD	9 groups of rep-PCR	All MDR Type 1 (79.8%) was the most common: major			Hui-Lan Chang, 2009

			, Taichung			clone that caused outbreak in this hospital			
4	*A. baumannii* 1997-1998 (2071) 1999-2005 (9021)	1985-2005	Tertiary hospital, northern Taiwan			1997-1998 nonsusceptible rate (2%) 1999-2005 (4.8%)			Wen-Tsung Lo, 2011
5	247 Acb 47.8% *A. baumannii* 32% *A. nosocomialis* 8.9% *A. pittii*	2007-2008	NTUH tertiary hospital	PFGE (77 non-baumannii)	No clonal spread in non-baumannii isolates	CR A. baum 44.3% A. noso 5.2% A. pittii 0%		A. baumannii bacteraemia was associated with a higher mortality rate (14, 30 days)	Yi-Chieh Lee, 2011
6	23 MDR AB	2009 (1 mo)	5 hospitals	PFGE	11 pulsotypes	5 pulsotypes were clonally spread among the 5 hospitals		OXA-23 (57%) ISAba1/OXA-51 (61%)	Ming-Feng Lin, 2011
7	92 *A. baumannii* (3.8%) From IAIs SMART	2006-2010	5 tertiary hospitals (Northern)			IMP-R 2006 (70%) 2007 (80%)		CRAB 2006 (70%) 2007 (81%) 2008 (58%) 2009 (16%) 2010 (25%)	Yu-Lin Lee, 2012
8	56 (3.2) *A. baumannii* in the Asia-Pacific region UTI SMART	2009-2010	38 medical centres form 10 countries			IMP-R ~64.3%			Po-Liang Lu, 2012
9	43 patients *A. junii* bacteraemia	2000-2010	NTUH				100% Carb S 35% colistin R		Tsai HY, 2012
10	42 clinical ACB isolates 29 environmental	2009 (a year0		PFGE, Rep-PCR	4 pulsotypes 4 types rep-PCR (same results w PFGE)	Type A (90.6%)	MDR 95.2% clinical 81.8% enviromental	OXA-23, OXA-51 (in MDR isolates)	Cheng-Mao Ho, 2013
11	134 MDR-AB	2007	3 hospital from north, south and east	PFGE	17, 23, 11 pulsotypes in each hospitals	High proportion of identical strains in eastern Taiwan	Resistance Colistin 10.4% Rifampicin 47.8% Tigeclycline 45.5%		Kai-Chih Chang, 2012
12	IMP-R isolates ACB 46 clinical/ 11 environmental	2003-22004	ICU veterans general hospital	PFGE	2 pulsotypes in both species	*A. baumannii* was closely related	*A. baumannii* (42/5) *A. nosocomialis* (4/6)	IS*Aba3*/OXA-58 22 clinical isolates AB All *A. nosocomialis* IS*Aba1*/OXA-51 in 21 clinical AB	Yi-Tzu Lee, 2012

13	13,811 *A. baumannii* infections	2003-2008	121 hospitals Nationwide			Significant increase in CRAB over that by all *A. baumannii* 14% in 2003 to 46% in 2008 The greatest: central Taiwan: 4% in 2003 to 62% in 2008		Use of anti-psuedomonal carbapenems was significantly correlated with the increase of CRAB	Chiu-Hsia Su, 2012
14	ACB isolates TSAR	2002-2010	26 hospitals (all 4 regions) Nationwide			IMP-R 2002 (3.4%) 2010 (58.7%) Susceptibility to polymyxin: 99.8% (remain high)			Shu-Chen Kuo, 2012
15	667 *A. baumannii*	1995-2012	Single hospital: north	PFGE	Heterogeneous	Susceptibility to carb decreased steadily 88.1% (2001-2003) to <25% (2010-2012) Remain susceptible to colistin Partially susceptible to tigecycline (80%)		IS*Aba1*/OXA-51 consistently identified (start in 2001) Tn2006, Tn2008 (first appeared 2007) 17.5% (2007-2009) to 50% (2010-2012) ISAba3/OXA-58, OXA-72, MBL genes: sporadic	Wen-Wei Ku, 2013
16	192 *A. baumannii*	2009	10 teaching hospitals			IMP-susceptibility 29%			Shio-Shin Jean, 2013
17	151 ACB isolates 88.7% *A. baumannii* 8% *A. pittii* 3.3% *A. nosocomialis*	2006 (3 mo)	25 medical centres	PFGE, MLST (Pasteur)	3 majors clones: ST2, CC2 (Pasteur)	26 isolates: CRAB 7.7% OXA-23 7.7% OXA-24 No IMP, VIM	One *A. nosocomialis* had OXA-51	1 CR *A. pittii* OXA-58 5 all CS but one had OXA-51	
18	65 *A. baumannii* 55.4% IMP-R	2009 (6mo)	2 regional hospitals	Rep-PCR	One major clone of IMP-R (84%) in the four regions	72% class 1 integrase genes			Ming-Feng Lin, 2013
19	Bacteraemia 42% *A. baumannii* 47.7% *A. nosocomialis* CRAB (71) CRNS (64)	2000-2008	Veteran generals hospital	PFGE	7, 14 pulsotypes	CRAB 1.4% colistin R 8.2% tige R	CRNS 45.3% colistin R No tige R	Significantly higher mortality rate with CRAB 40.8% vs 14.1% at 14-day	Ya-Sung Yang, 2013

20	136 IMP-R *Acinetobacter* spp.	2005-2007	Regional hospital	PFGE	56 pulsotypes	No predominant pulsotype		OXA-23 (19%), chromosomal with ISAba1 OXA-24 (1%) IS*Aba1*/OXA-51 (10/39) (26%)	Ming-Feng Lin, 2011
21	83 IMP-R *Acinetobacter* spp.	2007	3 hospitals	PFGE	51 pulsotypes	No predominant pulsotype		OXA-23 (32.5%) OXA-72 (6%) OXA-51-like were OXA-66	Han-Yueh Kuo, 2010
22	291 *A. baumannii*	2007 (4 mo)	10 hospitals	PFGE	2 major clones (77.8%)	142 (49%) IMP-R		27 (19%) OXA-72: predominance in southern Taiwan (22/38) All on 2 plasmid types	Shu-Chen Kuo, 2013
23	19 *A. pittii*	1999-2007	3 hospitals	PFGE	16 pulsotypes	No clonal relatedness	Carb-R 57.9% (11) All susceptible to colistin	IMP-1 (10) IMP-8 (1) OXA-58 (11) with IS*Aba3*	Li-Yueh Huang, 2010
24	1,265 *A. baumannii*	2007-2009	Chung Shan Med University, central Taiwan			15.18% MDR		96.35% (185/192) OXA-23 0.52% (1/192) OXA-24	Yang SC, 2010
25	62 *A. baumannii* (42 IMP-R)	2009	2 teaching hospitals	PFGE	41 types			IMP-R isolates Tn*2006* (28) Tn*2008* (6) IS*Aba1*/OXA-51 (8) IS*Aba3*/OXA-58 (2) IMP-1 (1)	Hao-Yuan Lee, 2012
26	32 XDRAB	2006 (2 mo)	4 ICUs in Tertiary hospital, southern Taiwan	PFGE	1 pulsotypes	Clonal spread caused outbreak		All OXA-72 (OXA-24-like), IS*Aba1*/OXA-51	Wei-Ru Lin, 2011
27	92 MEM-R	2005-2007		PFGE	11 pulsotypes 3 major pulsotypes	76% belonged to one of three pulsotypes		OXA-72 (59) (plasmidic) IS*Aba1*/OXA-51 (33) OXA-58 (2) OXA-23 (1) Loss of CarO doesn't produce a higher MIC	Po-Liang Lu, 2009

Thailand

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	13 CRAB	2006 (1 mo)	Regional hospital, North	PFGE	8 pulsotypes	2005 IMP-R 57% Genetically diverse, with some isolates were closely related		In 2004, OXA-72 chromosome-encoded was identified (GenBank AY739646) All OXA23 IMP (2) Both on plasmid	Niumsup PR, 2009
2	200 CRAB	2003, 2004, 2006, 2008	Siriraj Hospital	PCR-RAPD Plasmid profiling	3 RAPD types 10 plasmid profiles	I (20), II (16), III (1) 2 clones with OXA-23 wide-spread in this hospital		37 isolates: PCR All OXA-23/IS*Aba1*	Thapa B, 2010
3	83 *A. baumannii* 69% IMP-R	2005-2006 (3 mo)	Ramathibodi hospital	PFGE	36 types	3 predominant types Inter-intradepartmental spread		Class 1 integrase: all IMP-14 (1)	Kansakar P, 2011
4	115 *A. baumannii* COMPACT II Asia-Pacific study	2010 (4mo)	20 medical centres, 4 countries			CNSAB (73%) Philippines 25% Singapore 90.5% Thailand 76.3% Vietnam 89.5%			Kiratisin P, 2012
5	72 *Acinetobacter* bacteraemia	2005-2008	2 rural provinces			44% non-HCA risk factors: 56.3% Acb complex 43.8% (non-Acb) 56% HCA infections 79% Acb 21% non-Acb	Underline the need for clinician awareness of *Acinetobacter* spp. as a potential cause of CA bacteraemia in Thailand	Greater proportion of non-HCA infections were due to non-Acb complex	Porter KA, 2013
6*	2,130 *A. baumannii*	2003-2005	Songklanagarind			Outbreak of IMP-R AB in this hospital is asso with increasing ATB pressure, esp IMP, admission to ICR, RCU		Not significant difference in mortality rate between IMP-R AB and IMP-S AB	Jamulitrat S, 2007
7*	NARST	2000-2005	33 hospitals			CRAB 2000 (2.1%)			Apisarnthanarak A, 2009

			Nationwide			2005 (46.7%) Emergence of PDR AB			
8*	*Acinetobacter* 8,699 (in 2000) 14,071 (in 2005) NARST	2000-2005	28 hospitals			Same as 7			Dejsirilert S, 2009
9*	30 patients 300 environmental isolates	2008 (15 d)	BMA medical college	PCR-based typing (65 CRAB)	4 molecular types Type 1 (90.7%): found in almost all kinds of environmental samples	86.7%/ 66.7% of 30 patients had their environment contaminated with AB / CRAB	Enviroment contamination rates of AB/ CRAB were 18%/ 13%) The most contaminated sites: cupboards (26.7%) Bedrails, bed sheet s (20%) BP cuffs (16.7%)	Clonal spread of CRAB emphasise the importance of hand hygiene, contact precaution and patient's environment decontamination	Phumisantiphong, 2009
10*	114 *A. baumannii* 90% HAI 7.5% colonization 2.5% CAI		Phramongkutklao Hospital	PCR-based typing	6 molecular types	Type 2 (47.4%) most common and widely spread in 14 wards		67.5% MDR	Aimsaad L, 2009
11*	651 nosocomial pneumonia patients 198 (30.4%) *A. baumannii*					Most of *A. baumannii* resistant to many ATB including carb			Werarak P, 2012
12*	10,762 patients 7.3% HAIs	2011				*Acinetobacte*r spp.: most common organism			Rongrungruang Y, 2013

Asia-Pacific, WW

No.	Isolates	Year	Hospital	Typing Method	Clonality	Result	Non-baumannii	Resistance gene	Reference
1	492 IMP-non-susceptible AB		32 countries	DiversiLab, PFGE, Sequence group multiplex	8 groups (91%)	~50% (241) WW2		OXA-23 (32%) OXA-58 (22%) OXA-40 (8%) IS*Aba1*/OXA-51 (39%)	Higgins, 2010
2	*A. baumannii* with Intra-abdominal infections 2003-6 (173)	2003-2010	Asia-Pacific			IMP-non-susceptible 2003-6 (60.7%) 2007-10 (39.4%)		*bar chart of susceptibility to IMP with IAIs NZ, HK,	Yuag-Meng Liu, 2012

	2007-10 (312) SMART		Aus, China, HK, Malay, NZ, Phil, Spore, SK, TW, TH					TW, CH, KO in 2003-10	
3	411 *Acinetobacter* spp. (APAC) SENTRY	2004-2006	17 countries from Asia-Pacific			IMP/MEM-S 73.7/73%			Gales AC, 2006
4	1952 *Acinetobacter* spp. (APAC) SENTRY	2006-2009	12 countries APAC			IMP-S 2006 (59.4%) 2007 (69.4%) 2008 (48.6%) 2009 (37.4%) All (56.8%)		APAC showed the lowest rate of susceptibility to IMP	Gales AC, 2011
5	253 *A. baumannii* (93.7%) *A. nosocomialis* (10) *A. pittii* (5) >60% *A. baumannii* causing HAP were resistant to all antimicrobial agents except the polymyxins indicting, which indicates that the continuing increase of highly MDR-AB in Asia (Chung DR, 2011)	2008-2009	73 hospitals, 10 Asian countries	MLST (108 isolates tested)	36 STs	76.9% CC92 (83) from all 8 countries: 18 STs ST92 (19) (IN, MY, PH, TW, TH), ST195 (18) (TH, MY) are predominant CC108: prevalent in India (5) CC119 (3), CC254 (3), singleton (11)		82.5% CRAB (208) 108 tested: ISAba1/OXA-51 6.5 % (7) OXA-23 94.4% (102) OXA-24 6.5% (7) all from Taiwan All CC92 (47 tested): AbaR4-like (AbaR25-I most prevalent (34.8%))	Dae Hun Kim, 2013